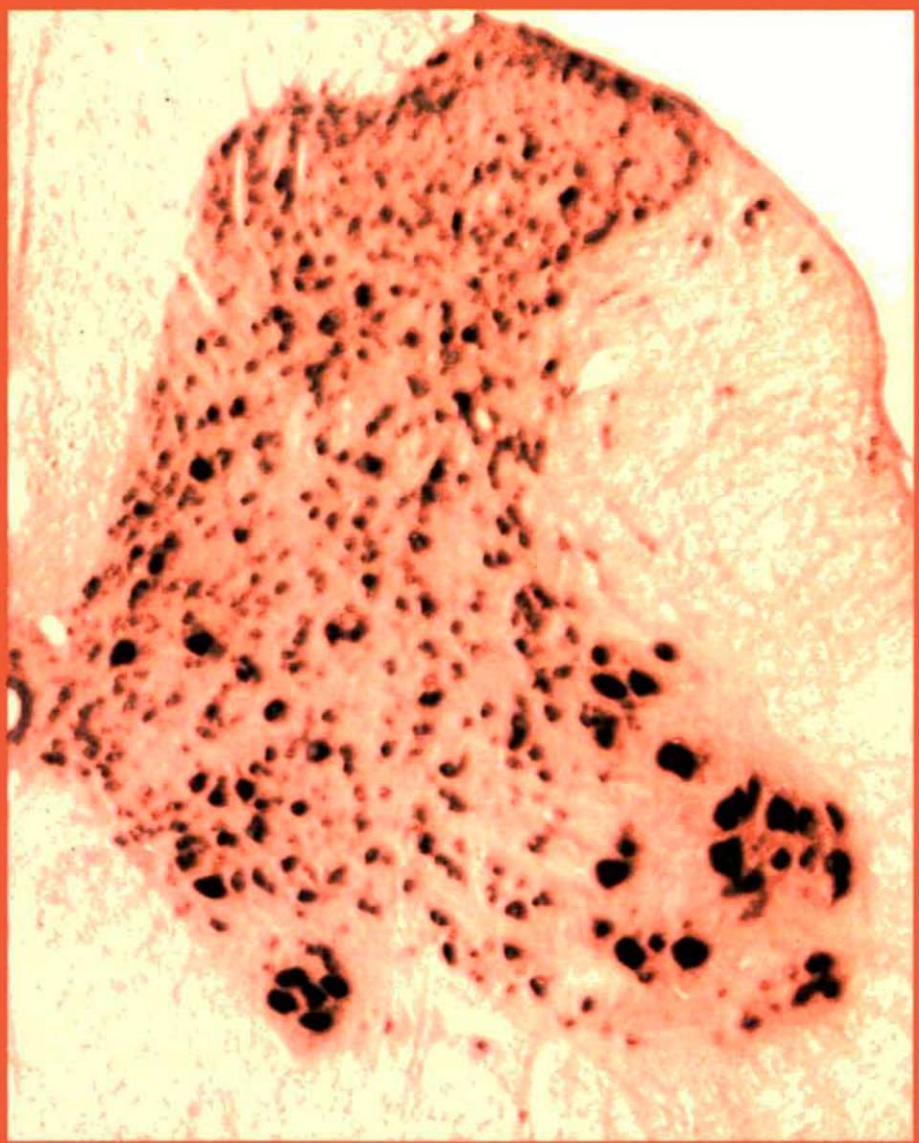
Acta Universitatis Szegediensis

Acta Biologica Szegediensis

Volume 45, Number 1-4, 2001



University of Szeged, Szeged, Hungary

Acta Biologica Szegediensis

Acta Biologica Szegediensis (ISSN 0324-6523), a member of the Acta Universitatis Szegediensis family of scientific journals (ISSN 0563-0592), is published yearly by the University of Szeged. Acta Biologica Szegediensis covers the growth areas of modern biology and publishes original research articles and reviews, involving, but not restricted to, the fields of anatomy, embryology and histology, anthropology, biochemistry, biophysics, biotechnology, botany and plant physiology, all areas of clinical sciences, conservation biology, ecology, genetics, microbiology, molecular biology, neurosciences, paleontology, pharmacology, physiology and pathophysiology, and zoology. Occasionally, Acta Biologica Szegediensis will publish symposium materials. Acta Biologica Szegediensis particularly encourages young investigators and clinicians to submit novel results of interest.

Submission of manuscripts

Manuscripts should be prepared in accordance with the Instructions to Authors published in each issue, also available at http://www.sci.u-szeged.hu/ABS, and submitted to:

Károly Gulya
Acta Biologica Szegediensis, Editorial Office
Department of Zoology and Cell Biology
University of Szeged
2 Egyetem u., POB 659, H-6722 Szeged, Hungary
Phone: 36 (62) 544-048, fax: 36 (62) 544-049
E-mail: gulyak@bio.u-szeged.hu

Correspondence relating to the status of the manuscripts, proofs, publication, reprints and advertising should be sent to:

Lidia Bakota
Acta Biologica Szegediensis, Editorial Office
Department of Zoology and Cell Biology
University of Szeged
2 Egyetem u., POB 659, H-6722 Szeged, Hungary
Phone: 36 (62) 544-125, fax: 36 (62) 544-049
E-mail: bakotal@bio.u-szeged.hu

Subscriptions

Acta Biologica Szegediensis is published yearly in four issues per volume. All subscriptions relate to the calendar year and must be pre-paid. The annual subscription rate is currently 30 USD and includes air mail delivery and handling.

Acta Biologica Szegediensis is indexed in BIOSIS Database, EMBASE, Excerpta Medica, Elsevier BIOBASE (Current Awareness in Biological Sciences) and Zoological Record.

The Table of Contents for the current issue and those for previous issues can be found at http://www.sci.u-szeged.hu/ABS.

ACTA BIOLOGICA SZEGEDIENSIS VOLUME 45(1-4), 2001

Table of Contents

Distribution of DIG-labeled CaM I specific riboprobe in the lumbar spinal cord of the adult rat. Color in situ hybridization technique (Cs. Szigeti, Dept. Zoology & Cell Biology, University of Szeged)

Volume 45(1-4):1-11, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

REVIEW ARTICLE

Avian adrenal medulla: cytomorphology and function

Asok Ghosh, Stephen W. Carmichael[1]*, Monisha Mukherjee

Department of Zoology, University of Calcutta, Calcutta, India, [1]Department of Anatomy, Mayo Clinic/Foundation, Rochester, Minnesota, USA

ABSTRACT The purpose of this review is to explore the world literature on the avian adrenal medulla from the last 20 years. Unlike the mammalian adrenal medulla, the adrenal gland in birds has chromaffin cells mixed with cortical cells. Studies have investigated the ultrastructure (both transmission and scanning electron microscopy), biochemistry, and physiology (particularly interactions with other endocrine glands) of the avian adrenal medulla. Although progress has been made, it is apparent that research on the avian adrenal medulla still lags behind work on the mammalian organ.

Acta Biol Szeged 45(1-4):1-11 (2001)

KEY WORDS

adrenal medulla
birds
morphology
function

The adrenal glands of birds, like those in mammals, are paired yellow- or orange-colored pear- or triangle-shaped glands that are next to the kidneys. The intermingling nature of cortical and medullary components constitutes a major characteristic of avian adrenal medulla (Vestergaard and Willeberg 1978). This is in sharp contrast with its mammalian counterpart, where the medulla always occupies the central portion and remains encircled by the adrenal cortex with its three concentric zones.

Compared to the adrenal cortex, the avian adrenal medulla was a much-neglected subject until the 1950s. Ghosh (This will refer to Asok Ghosh. His son, also an active scientist in the field, will be referred to as Subho Ghosh) and his collaborators pioneered efforts to elucidate various aspects of avian endocrinology, particularly the adrenal gland (Ghosh 1977). This first review article on this subject was published by Ghosh, that covered works up to 1977. Later, a second review by Ghosh in 1980 gave literature coverage from 1978 to 1980. The present article will emphasize the world's literature on the avian adrenal medulla from 1981 through 2000.

Ghosh and his collaborators (see Ghosh 1977, 1980) studied the relative proportion of epinephrine (E)- and norepinephrine (NE)-secreting cells and cellular areas in the adrenal medulla of a number of birds representing several orders and families. The data shows extreme variation in the relative concentration of E and NE cells in avian adrenal medulla that is not seen in other vertebrate groups. Light and ultrastructural studies (Subho Ghosh 1977) revealed that the orders with a more primitive ancestry contain more NE cells, whereas the more recently evolved birds possess more E cells in the adrenal medulla. This profound variation of medullary E/NE ratio in birds suggests a distinct evolutionary pattern (Ghosh 1977, 1980). The avian phylogeny used in this study was essentially based on palaeontological evidences (Gregory 1957). We feel that our "claim" of hormonal taxonomy is to be re-examined in view of cladistic (Cracraft 1988) and molecular (Mindell 1997) analyses of avian taxa (also Bhattacharyya, B. 1999, personal communication). Later Mahata and Ghosh (1986a, c, 1988) reported that NE and E contents vary not only between birds of different phylogenetic groups, but also with the age of birds of the same species.

Accepted May 18, 2001
*Corresponding author. E-mail: carmichael.stephen@mayo.edu

I. Morphological considerations

A. Development

Elegant work from Unsicker's laboratory (Ross et al. 1995) shows that by embryonic day 15 (E_{15}) sympathetic ganglia of chick embryos contain a glucocorticoid-responsive progenitor population that can differentiate into medullary cells. Another important work by Sanchez-Montesinos et al. (1996) examined the development of the chick sympathoadrenal system by identifying antibodies that recognized signal molecules. Three expression patterns were found in the developing adrenal gland defining early permanent markers (chromogranins A and B, GaO, TH, and galanin); others (DbH, somatostatin, enkephalin, secretogranin 11, NPY, and PNMT) follow as development proceeds.

B. Subcellular morphology

During the last three decades, the cytophysiology of the avian adrenal medulla has been thoroughly worked out by Ghosh and his associates by means of histological, histochemical (Ghosh 1980), transmission (TEM; Ghosh et al. 1996a), and scanning (SEM; Guha et al. 1990) techniques.

Maitra and Ghosh (1980) differentiated the two types of tinctorially different adrenomedullary cells in the plum-headed parakeet (*Psittacula cyanocephala*) by application of a modification of the Wood (1963) technique. The E cell islets become densely granulated and reddish-brown colored, whereas the NE cells are homogeneously yellowish in color, smaller in size, and fewer in number. The E- and NE-secreting cells are also different on the basis of their morphology, size, opacity, and fine structure of the vesicles (see Unsicker 1973a, b, c; Ghosh and Guha 1988).

Unsicker (1973a, b, c) reported that E and NE cells could be distinguished from each other on the basis of their structure and granulations. E cells exist in all avians, but in corvids (includes crows, ravens, rooks, magpies, and jackdaws) and in some passers, they possess low electron dense vesicles and lack complete membranes. The average size of NE vesicles in domestic chicken (*Gallus domesticus*) is greater (224 nm) than the E (168 nm) vesicles (Coupland 1971). The E and NE vesicles follow a similar pattern in the rose-ringed parakeet (*Psittacula krameri*; Carmichael et al. 1983) and in duck and geese (Guzsal and Hassan 1975). In the migratory snipe (*Capella gallinago*), only NE types of chromaffin cells are observed (Carmichael et al. 1985). In pigeons (*Columba livia*) and bulbuls (*Pycnonotus cafer*), two distinct cell types are present. Although the vesicles of cells in pigeons do not manifest size difference, in bulbuls the E vesicles are comparatively large (Ghosh and Guha 1988). Cuello (1970) found a wide range of sizes of NE vesicles (80-500 nm) in the gentoo penguin (*Pygoscelis papua*). The TEM studies by Guzsal and Hassan (1975) reveal presence of the so-called "dark cells" and the transitory sympathicoblasts in the adrenal medulla of ducks and geese.

TEM studies of adrenal gland enables differentiation of the E and NE cells by their morphology, size, opacity, and fine structure of the vesicles (vide supra). But, in contrast to the mammalian adrenal medulla, it has not been resolved whether E and NE cells originate from the same, or two separate cell types, of the adrenal medulla (Ghosh and Guha 1988). Recently, an electron microscopic study of the chromaffin cells in the adrenal gland of 13 species of birds from the Indian subcontinent was performed by Subho Ghosh et al. (1996a). The study revealed that the cytoarchitecture of the adrenal medulla is similar in many respects in all the birds. However, differences were noted in the density of chromaffin vesicles, subunit organization in certain vesicles, compartmentalization of electron dense vesicles and electron lucent vesicles in a single cell, presence of four cell types (dark, clear, vacuolated, and mixed). There were also some differences among species in the prominence of the interface between the cortical and medullary tissues and in the frequency of exocytotic profiles.

The three-dimensional surface information of the adrenomedullary parenchymal cells is available from studies on woodpecker, kingfisher, parakeet, and common snipe (Guha et al. 1990). The presence of blebs, cords, globules, granular particulates, filamentous, and coral-like aggregates on the adrenal surfaces also show great inter-specific variations. In the rose-ringed parakeet, SEM observations reveal densely granulated medullary cells, and a cytoplasm containing a single central or eccentric nucleus. Prominent microvillus-like projections are seen that appear to connect nucleus with surrounding cytoplasm. The vesicles are oval, round, or cylindrical and are frequently seen to fuse with one another. Also there occurs conglomeration of bleb-like structures in the blood spaces. The endothelium has rufflings and breakages at points from which blebs appear to be discharged (Carmichael et al. 1983).

C. Immunocytochemistry

By immunocytochemistry, it is suggested by Ohmori et al. (1997) and Ohmori (1998) that serotonin, galanin, cholecystokinin, met-enkephalin, somatostatin, and natriuretic polypeptides exist in chicken chromaffin cells, in addition to E and NE. Different biogenic amines and neuropeptides localized in medullary cells may regulate corticosterone secretion from interrenal cells via a paracrine mode of communication (cf. Nussdorfer 1996).

II. Biochemical aspects

A. Biosynthesis

Aspects of biosyntheses have been discussed earlier (Ghosh 1977, 1980). The dopamine antagonist haloperidol stimulates aldosterone and corticosterone secretion in rats (Goebel et al. 1992) and inhibits specific high affinity binding sites in the membrane of adrenal medulla (Rogers et al. 1989). Recent work in rats by Mukherjee et al. (1995) shows significant release of corticosterone following haloperidol treatment. The catecholamine content of adrenal medulla remains non-responsive, probably due to the specific blocking of the D_2-dopamine receptors. This aspect has remained unresolved in avians and needs intense attention.

Studies on rats show that the enzyme phenylethanolamine *N*-methyltransferase (PNMT) is localized in the E cells only. Using an immunocytochemical technique, our laboratory group (Guha et al. 1992) indicates that PNMT location is similar at least among phylogenetically close avian species. Unlike mammals, no particular cell specificity for this enzyme exists in birds. From their studies, it may be conjectured that PNMT does not act as a marker enzyme in differentiating E and NE cells. Rather, it is presumed that PNMT exists in all adrenomedullary chromaffin cells of avians, and only in some cells it exists in an active form and plays role in conversion of NE to E.

Ascorbic acid acts as co-factor in the β-hydroxylation of dopamine, which is the rate-limiting step in the synthesis of

NE (Subramanian 1980). Experimental administration of vitamin C in the red-vented bulbul (*Pycnonotus cafer*; Ghosh and Chatterjee 1985) shows significant rises of NE and fall in E within the adrenal medulla. This indicates that vitamin C lowers glucocorticoid levels necessary for methylation process (cf. Pohorecky and Wurtman 1971). In experimentally scorbutic bulbuls (Chatterjee and Ghosh 1982), E content of adrenal medulla becomes conspicuously high as compared to its non-methylated counterpart, NE. In avians, vitamin C also plays role in the release of catecholamines (mainly NE) and possibly, like mammals, it is also involved in the catabolism of NE to normetanephrine (Ghosh and Chatterjee 1986).

B. Phospholipid profile

Although study of adrenal phospholipid has been fairly well known in mammals, a systematic work in this field was not documented in avians until the early sixties (Ghosh 1962). Recently, the phospholipid content of the adrenal of nine bird species belonging to different phylogeny was worked out by Chakrabarti et al. (1996). Eight different adrenal phospholipid fractions (phosphatidic acid, cardiolipin, phosphatidyl ethanolamine, phosphatidyl glycerol, lecithin, sphingomyelin, lysolecithin, and phosphatidyl inositiol) were identified. The study shows that percentage composition of phosholipids, excepting phosphatidyl inositol, does not manifest a difference among species. The major phospholipids of the medulla, phosphatidyl ethanolamine, lecithin, and sphingomyelin, are suggested to play significant role in the secretory process of the chromaffin cells.

C. Release mechanisms

It is well established in mammals that the mode of adrenomedullary secretion is exocytosis (Diner 1967). In birds, however, the process of medullary granular extrusion is not yet understood as clearly (Carmichael et al. 1985, 1987). Unsicker (1973a, b, c) studied the ultrastructure of the adrenal medulla of a number of avian species and found that only the chaffinch (*Fringilla coelebs*) exhibits exocytosis. In a comparative TEM study of the adrenal medulla of thirteen subtropical birds, Subho Ghosh et al. (1996a) observed "exocytotic profiles" only in woodpecker (*Dinopium benghalense*) and kingfisher (*Halcyon smyrnensis*). Very recently, Subho Ghosh et al. (1999a) further found no "exocytotic figures" of the adrenomedullary chromaffin vesicles after treatment of the domestic pigeon (*Columba livia*) with lithium chloride (LiCl). It is, therefore, still not proven that exocytosis is the only mechanism of secretion of chromaffin vesicles in birds, contrary to what had earlier been indicated by Chungsmarnyart and Fujioka (1982). The intricate problem of the release mechanism of avian adrenomedullary chromaffin vesicles has yet to be resolved.

For example, it has been found that $G_{\alpha 0}$ protein is associated with the mammalian chromaffin cell membranes. Activation of this protein causes inhibition of exocytosis. When the $G_{\alpha 0}$ protein is inactivated, exocytosis takes place (Vitale et al. 1994). Studies such as this needs to be done to better appreciate if exocytosis is the mechanism of secretory release in birds.

C. Role of monoamine oxidase isozymes

The recent study of Subho Ghosh et al. (1995) reveals that the adrenal medulla of pigeons possess 60% type A and 40% type B isozymes of monoamine oxidase (MAO). Application of pargyline (a MAO-B inhibitor) or clorgyline (MAO-A inhibitor) together with guanithidine sulfate (an adrenal catecholamine releaser) in pigeons reveal that guanithidine action on adrenal medulla becomes more prominent when the MAO-B isozyme is inhibited, suggesting that MAO-B suppresses catecholamine release.

D. Role of lithium

Lithium (Li) has a well-documented efficacy in psychiatric disorders. The salts of Li act as a mood-stabilizer and thus is used for treatment of manic depression. Although the effects of Li on peripheral endocrine glands have been elucidated in recent years, literature on its role on the adrenal gland is scarce. Recently, Subho Ghosh et al. (1997b) have shown that in pigeons (*Columba livia*) Li reduces tissue stores of catecholamines significantly by activating MAO. Pargyline completely blocks Li-induced depletion of catecholamines from pigeon adrenal medulla, whereas clorgyline fails to exhibit a response. It is thought that in pigeon, the inhibitor of MAO-A and -B isozymes act in a different pattern. Li reduces the catecholamine content of adrenal medulla by utilizing MAO-B.

An integrated approach involving ultrastructural (TEM), histologic, and biochemical probes were employed to study the effect of Li on the pigeon adrenal medulla (Subho Ghosh et al. 1999a). Both E and NE vesicles were found to decrease after treatment with Li. The overall degeneration, *i.e.* atrophy, of the medulla along with significant depletion of the catecholamines (E and NE) was observed after lithium chloride (LiCl) administration. It was shown that LiCl had a degenerative effect on the pigeon adrenal medulla.

Of the eight phospholipid fractions studied, only two fractions (phosphatidyl glycerol and phosphatidyl ethanolamine) showed significant changes (increase and decrease, respectively) as a result of LiCl treatment. Absence of any detectable change in phosphatidyl inositol content of Li-treated birds suggested different modes of Li action in the adrenal medulla of birds and mammals (Chakrabarti et al. 1999).

The effect of LiCl on the adrenal hormones was studied during experimental hyperthyroidism in the pigeon. It revealed that experimentally altered thyroid function had no effect on the adrenomedullary catecholamines, but significantly increased the corticosterone level. The investigation suggested that lithium has a different effect on the adrenal cortex and medulla during hyperthyroidism (Subho Ghosh et al. 1999b).

III. Physiological considerations

A. Neural regulation of catecholamine release

(a) Role of the splanchnic nerve

The chromaffin cells of adrenal gland in homeothermic vertebrates are mainly innervated by preganglionic sympathetic nerve fibers that travel in a splanchnic nerve (Coupland 1971). Mahata and Ghosh (1986b) presented the first evidence of neural regulation of catecholamine release in birds (pigeon, *Columba livia*). They showed that splanchnic denervation of the adrenal medulla has no significant effect on adrenal weight, medullary histology, NE fluorescence, and catecholamine content in a species of bird (Mahata and Ghosh 1986b). However, splanchnic nerve modulates adrenomedullary functions under various experimental conditions. Splanchnic denervation in the pigeon (*Columba livia*) and duck (*Anas querquedula*) fails to affect reserpine-induced depletion of E, whereas it prevents reserpine-induced depletion of NE only at a low dose of reserpine (Mahata and Ghosh 1989). Unilaterally denervated pigeons treated with reserpine and protein hormones show that the splanchnic nerve modulates synthesis, release, and resynthesis of catecholamines in the avian adrenal medulla (Mahata and Ghosh 1991a). The presence of adrenergic nerve fibers in the avian adrenal medulla has been demonstrated from exhaustive work on fifteen avian species. All of these studies indicate that post-ganglionic adrenergic nerve fibers are exclusively associated with NE cells, and thus the splanchnic nerve plays a vital role in the synthesis of NE, but not E (Mahata and Ghosh 1988).

To determine neural regulation of adrenomedullary functions in birds, Mahata and Ghosh (1989) applied reserpine at three doses (0.05, 0.2, and 0.8 mg/100 g body weight) to seven unilaterally splanchnic denervated birds. Results show that reserpine at high doses cause 66-92% depletion of total catecholamines from both innervated and denervated gland in all species investigated. A low dose of reserpine depleted 40-84% of catecholamines from the adrenal gland in passerine birds (common myna and bulbul) whether their nerve supply was intact or not. However, in non-passerine birds (duck and pigeon), a low dose of reserpine depleted 66-71% of NE from innervated gland as compared to only a 2-13% reduction from denervated glands. In these birds, E depletion was 60-85% that was independent of neural regulation. The findings suggest that at high dose of reserpine, depletion of catecholamines is controlled by some non-neurogenic mechanism. At a low dose of reserpine, the splanchnic nerve probably modulates depletion of catecholamines from the adrenal medulla of non-passerine birds, whereas the same is governed by some non-neurogenic mechanisms in the passerine birds. Role and Perlman (1983), Wakade and Wakade (1983), Marley et al. (1985), and Khalil et al. (1986a,b, 1987) also reported that in mammals both neural and non-neural mechanisms are involved in release of catecholamines from the adrenal medulla.

A study by Mahata and Ghosh (1991a) has demonstrated that administration of steroid hormones (corticosterone, dexamethasone, deoxycorticosterone, progesterone, testosterone, and estradiol) on unilaterally splanchnic denervated pigeons causes significant changes in catecholamine content. Specifically, the E content between the innervated and denervated glands is markedly different. From this finding it becomes evident that the splanchnic nerve regulates steroid-induced alterations of E content in the pigeon. The results further reveal that the glucocorticoid hormones augment reserpine-induced resynthesis of catecholamines in the innervated glands. These results confirm that the splanchnic nerve is essential for the synergistic action of glucocorticoid- and reserpine-accelerated resynthesis of catecholamines.

(b) Role of the vagus nerve

Pilo and his collaborators (1984) have demonstrated an influence of the vagus nerve on the adrenal functions in homeothermic vertebrates. Histomorphologic studies carried out on adrenal glands of bilaterally vagotomized pigeons show that the adrenal gland, to some extent, is dependent on the vagus for its structural and functional integrity. Vagotomy results in an increase of adrenal weight. Both acetylcholinesterase and ascorbic acid levels become significantly lower in the adrenal gland of vagotomized birds. It also leads to a reduction in the proportion of medullary cells. It can be surmised that both in birds and mammals vagotomy causes a hypertrophy of cortical tissue and an atrophy of chromaffin cells.

B. Role of adrenal medulla in carbohydrate metabolism

The participation of the adrenal medulla in carbohydrate metabolism has been well elucidated in mammals. On the contrary, only a few references exist on the role of E and NE from the adrenal medulla in the avian glycemic response. Chattopadhyay (1986) performed investigations on the role of adrenomedullary hormones in the regulation of glucose homeostasis in birds. Starvation experiments on birds of different food habits as the rose-ringed parakeet (*Psittacula krameri*, which is graminivorous), the house crow (*Corvus*

splendens, which is omnivorous), and the spotted owlet (*Athene brama*, which is carnivorous) show simultaneous depletion and release of catecholamines and glycogen. Similarly, glucose loading in pigeons (*Columba livia*) increases glycogen level and store of catecholamines in the adrenal medulla. Administration of various autonomic effector agents that modify adrenal catecholamine levels (E, NE, isoproterenol and acetylcholine), causes simultaneous change in the glycemic response of the pigeon. The study further shows that β-receptors mediate the effect of catecholamines on carbohydrate metabolism in pigeons.

C. Role of the adrenal medulla in lipid metabolism

Sturkie (1965) reported that the adrenal medulla is less important in the mobilization of free fatty acids (FFA) in birds than in mammals. It appears that glycogen may be the prime lipolytic factor in birds.

A study by Sen and Bhattacharya (1984) on two avian species with distinct physiologic and metabolic differences, show that 18 hour fasting in the graminivorous rose-ringed parakeet (*Psittacula krameri*) causes a significant drop in plasma glucose and tissue glycogen and a simultaneous increase in plasma glucose, tissue glycogen, and plasma FFA levels. It also indicates an involvement of the adrenal glands in restoring metabolic homeostasis. Similar changes in glucose, glycogen, and FFA levels are found in the carnivorous collard scops owl (*Otus bakkamoena*), also. However, when the fasting birds are subjected to a single dose of E, NE, insulin, or glucagon (that are directly involved in carbohydrate and lipid metabolism), the pathway of response becomes altered. This apparently denotes some physiological and metabolic differences amongst various species of birds.

D. Response to stress

A stress-induced osmotic alteration leads to changes in the adrenal gland functions in birds. However, the response of the adrenal medulla to osmotic stress is highly variable. The findings of Ghosh and Sitaraman (1975), and later by De and Ghosh (I993), reveal that E possibly is the hormone that mediates stress in the pigeon (*Columba livia*), as also reported earlier by Kobayashi et al. (1980) in the Japanese quail (*Coturnix coturnix japonica*). In the house sparrow (*Passer domesticus*), both E and NE are required for counteracting the stress situation (De and Ghosh 1993).

Water deprivation induced stress in three species of munias adapted to different habitats (*i.e.* swampy, *Lonchura malacca*; grassland, *Lonchura malabarica*; and arid, *Lonchura punctulata*) yields differential responses (Subho Ghosh et al. 1993). Those adapted to swampy and grassland areas show significant depletion of catecholamines, whereas those inhabiting arid lands exhibit depletion of NE only. Differential secretion of E and NE in munias inhabiting swampy and arid habitats are related with the degree of osmotic and/or hypovolemic cues as determinant factor(s) for triggering specific sites within the diencephalon (Banerjee et al. 1994). The details of stress adrenal interactions in birds will be discussed below.

E. Stress recovery action of vitamin A

In mammals, vitamin A plays a role in ameliorating the effects of stress (Olson 1984). However, no such information was available until 1992, when Ghosh et al. (1992) first explored this in birds. Chronic vitamin A therapy causes hyperglycemia and rise in glandular E in pigeons (*Columba livia*), but birds exposed to psychophysical stress (cold-wet immobilization) become normoglycemic with a rise in NE. It is presumed that vitamin A modulates the synthesis and release of catecholamines under conditions of stress in pigeons.

IV. Endocrine interactions

A. Adrenal corticomedullary relationship

The relationship between adrenal medulla and cortex is well established. Mammalian glucocorticoids regulate the activities of tyrosine hydroxylase (TH; Lucas and Thoenen 1977) and PNMT (Wurtman and Axelrod 1965; Ciaranello 1978). Aldosterone treatment also elevates PNMT activity, but its potency is less than that of the glucocorticoids (Wurtman 1966). Glucocorticoid administration increases NE content in the chick (Wasserman and Bernard 1971) and E content in pigeon adrenal glands (Chaudhuri et al. 1966; Sitarainan and Ghosh 1977). Recently Mahata and Ghosh (1991a) have proved the effect of cortical steroids (corticosterone, dexamethasone, and deoxycorticosterone) on the neural regulation of adrenomedullary catecholamines. The study indicates that splanchnic nerve is essential for the synergistic action of glucocorticoids and reserpine in augmenting resynthesis of CA in pigeons.

B. Relationship with the endocrine pancreas

Avian adrenal medulla and pancreatic hormones hold a close relationship. Mahata and Ghosh (1986b) have demonstrated that the splanchnic nerve modulates insulin-induced depletion of NE in pigeons (*Columba livia*). Administration of insulin (4 IU/100 g body weight) caused depletion of NE from both intact and denervated adrenal glands in both newly hatched and adult pigeons. This treatment failed to modulate E content of the innervated and denervated glands. This indicates that the splanchnic nerve and age have definite influences on the effect of insulin on adrenal medulla. The study by Mahata et al. (1990a) on the effect of insulin on adrenomedullary catecholamine contents in a number of avian species (chicken, pigeon, parakeet, common myna, red-

vented bulbul, and babbler) points out that, with the exception of the babbler and parakeet, the action of insulin on avian adrenal medulla follows the mammalian pattern closely.

Except in pigeons (*Columba livia*) and red-vented bulbuls (*Pycnonotus cafer*), insulin- produced hypoglycemia alters, the catecholamine content of the avian adrenal medulla (Carmichael et al. 1987). Therefore, like mammals, adrenomedullary response depends on hypoglycemia also in birds (Mahata et al. 1990a). Administration of insulin in unilaterally splanchnic denervated pigeons at various time intervals show that whereas the splanchnic nerve stimulates the resynthesis of NE, synthesis of E is increased by insulin. Studies further show that insulin acts synergistically with reserpine to accelerate resynthesis of catecholamines in birds (Mahata et al. 1990b).

Glucagon administration in intact and splanchnic denervated pigeons also indicates that the splanchnic nerve regulates the release and/or resynthesis of catecholamines that are induced by glucagon (Mahata and Ghosh 1991).

C. Relationship with the pineal gland

Ultrastructural observations support the concept that adrenomedullary function, including mitotic activity, is controlled, at least temporarily, by the pineal gland (Kachi et al. 1988). In birds, the release of melatonin from the pineal is low during daytime (Takahashi et al. 1980). However, administration of melatonin during this period modulates the catecholamine content of avian adrenal medulla (Mahata et al. 1988). Later, Mahata and De (1991) found that the action of melatonin varies with age, species, dosage, and time of the day and that it modulates both NE and E contents of the adrenal medulla.

D. Relationship with the thyroid gland

The relationship between the adrenal medulla and thyroid gland in birds so far has remained comparatively less studied than in mammals. As mentioned earlier (Ghosh 1980) the work of Bhattacharyya (1971) on pigeon has proved that experimental hypo- and hyper- thyroidal drugs failed to alter adrenomedullary physiology and exogenous catecholamine administration had no effect on the thyroid gland. However under stressful situations, thyroid gland and adrenal medulla show striking synergism to counteract the effect of stress.

Bobek et al. (1996) analyzed the role of reverse triiodothyronine (rT_3) in heat stressed immature chickens. When injected subcutaneously, rT_3 (14 mg/100 g body weight) aggravates heat stress symptoms and increases plasma levels of corticosterone, catecholamines, and free fatty acids in chickens.

E. Relationship with the gonads

Application of gonadal steroids on the unilaterally splanchnic denervated pigeons results in an increase of E content of the adrenal gland (Mahata and Ghosh 1991). This finding indicates that 1) steroid hormones significantly alter adrenomedullary catecholamine content, and 2) changes of E content induced by steroid hormones are modulated by the splanchnic nerve.

Earlier studies by Pal and Chatterjee (1985) show that in the pigeon, testosterone application along with the corticoid inhibitor metapirone, has no effect on adrenomedullary catecholamines. On the contrary, testosterone treatment in metapirone pre-treated intact pigeons causes an increase of E. Perhaps testosterone influences methylation of NE, particularly in glucocorticoid-deficient pigeons.

The catecholamine-containing nerves of the testes of male birds exert an influence in regulating the functional state of the organ (Ljunggren 1969). Research from this laboratory by Mukherjee (1985, 1988) show that chronic applications of catecholamines and 6-hydroxydopamine (which causes "chemical sympathectomy"; Malmfors and Thoenen 1971) to the male weaver bird (*Ploceus philippinus*) produce extreme testicular degeneration. The unaffected hormonal content of the adrenal medulla under all these experimental conditions proves non-participation of the gland in controlling gonadal status, at least in this avian species.

In pigeons (*Columba livia*), however, a direct role of adrenomedullary catecholamines on testicular physiology was observed (Subho Ghosh et al. 1997a). Guanithidine treatment (which causes "chemical medullectomy") led to testicular degeneration with a concurrent fall in adrenal E and NE contents. Thus, differential responses exist in birds regarding relationship between gonad and adrenal medulla. In the blossom-headed parakeet (*Psittacula cyanocephala*), a phase-dependent relationship exists between adrenomedullary catecholamines and testicular cycle (Maitra and Ghosh 1982).

Studies on annual gonadal activity cycle of some common birds have given distinct indications of a positive correlation with catecholamines in the adrenal medulla. This was found in the Swedish wood pigeon (*Columba palumbus*), rose-ringed parakeet (*Psittacula krameri*), and blossom-headed parakeet (*Psittacula cyanocephala*; Maitra and Dey 1992). Interestingly, the gonadal phase and adrenal catecholamines are out of phase in the domestic pigeon (*Columba livia*) and lal munia (*Estrilda amandava*; Chakravorty et al. 1985).

In fowl, the weaver bird, and the tree pie (*Dendrocitta vagabunda*), adrenomedullary catecholamines exerted a negative influence on gonadal physiology. Mallick and Sarkar (1985) noticed parallelism between the testicular and adrenomedullary functions in the common myna (*Acridotheres tristis*).

Differential gonadal responses of parakeet and weaver bird to a particular photoperiod (22 light:2 dark) during

different phases of the annual gonadal cycle suggest the existence of an endogenous circennial periodicity in these birds. This finding suggests that in these sub-tropical birds, the duration of daily photoperiod possibly acts in the role of an entraining agent (also referred to as Zeitgeber). In the parakeet, entrainment of the photoperiod does not involve the adrenal glands in the mechanism that controls annual testicular cycle. The finding by Subho Ghosh et al. (1996b) shows that in the common myna (*Acridotheres tristis*), both day length and humidity control the testicular cycle. This subtropical avian species maintains a parallel type of adrenocortical-gonadal relationship. However, adrenomedullary hormones are also involved in the regulation of reproduction.

F. Relationship with neurohypophyseal hormone

The physiologic relationship between vasopressin and adrenal medullary catecholamines has been explored relatively recently. Nussey et al. (1987) first demonstrated that although arginine vasopressin (AVP) has no effect on basal catecholamine release from bovine chromaffin cells, it inhibited acetylcholine and nicotine-stimulated NE and E release in dose-related manner. Porter et al. (1988) have reported that AVP stimulated secretion of catecholamines from the rat adrenal gland perfused in situ.

Mahata and Ghosh (1991) first demonstrated the role of vasopressin in the avian adrenal medulla. Intraperitoneal injections of lysine vasopressin into unilaterally splanchnic denervated pigeons showed that in innervated gland, vasopressin caused 59-74% decrease of NE when compared to the denervated gland. This supports a role for the splanchnic nerves in preventing vasopressin-induced NE release. On the other hand, the splanchnic nerve has no effect on vasopressin-induced release of E. The resynthesis of both NE and E took place 144 hrs and 216 hrs after the injections, respectively. This points out that like mammals and birds, vasopressin possibly augments the release of ACTH that results in an accelerated resynthesis of both E and NE (Mahata and Ghosh 1991).

G. Role of prostaglandins in catecholamine action

Though extensive work has been done on the effect of prostaglandins (PG) on the adrenal medulla of mammals, findings relating to the regulatory role of PG on the hormonal release in birds are very limited (Das Adhikari and Guha 1991). Sengupta et al. (1994) and Sarkar et al. (1996) from our laboratory have demonstrated that chemically and structurally different PG inhibitors (indomethacin and ibuprofen) differ considerably in their mode of action on the adrenal hormonal profile during stress conditions in the same avian species. Treatment with indomethacin (a methylated indole derived PG blocker) exhibited no perceptible effect on either E or NE during acute formalin stress in the pigeon (*Columba livia*; Sengupta et al. 1994). On the other hand, Sarkar et al. (1996) observed that administration of ibuprofen (a propionic acid derived PG inhibitor) under acute stress condition significantly reduced the adrenal NE but had no influence on E. More recently, Sarkar et al. (1999) studied the effect of other PG inhibitors with different chemical natures such as aspirin (acetyl salicylic acid), mefenamic acid (fenamates), diclofenac (phenylacetic acid derivative), and piroxicam (an oxicam derivative) on the avian adrenal hormonal physiology during stress (see Table 1).

From the results of these studies it appears that aspirin, piroxicam, and diclofenac fail to alter the effects of formalin stress on the adrenal hormonal profile, whereas mefenamic acid accentuates the effects of the stress. Our studies reveal that chemically and structurally different PG inhibitors show diverse action in the same avian species under similar stress conditions. In this context, it seems worthwhile to explore which specific series of PG are inhibited by the prostaglandin blockers used.

H. Involvement of prolactin

The involvement of prolactin in maintaining electrolyte balance in avians is well documented by Mukherjee et al. (1993). The treatment in parakeet (*Psittacula krameri*) shows perceptible stimulation of the adrenal cortex and chromophobe cells of the adenohypophysis, but fails to modulate adrenomedullary functions at both cytologic and hormonal levels (Mukherjee et al. 1990). In the rat, a dopamine antagonist (haloperidol) induces adrenocortical hypertrophy but fails to generate an adrenomedullary response (Mukherjee et al. 1995).

I. Adrenal medulla — thyroid relationship

It becomes quite evident from the findings of Subho Ghosh et al. (1996b) that a parallel relationship exists between reproductive phases of the common myna (*Acridotheres tristis*) and levels of the hormones, *viz.* medullary E and NE, and T_3 and T_4.

J. Role of the bursa of Fabricius

The role of the bursa of Fabricius (*bursa fabricii*) during stress-induced modulation of adrenomedullary catecholamines has been studied by Mahata et al. (1990c). Studies on bursa-intact and bursectomized chicks exposed to cold-wet immobilization (CWI) stress demonstrate for the first time that the bursa modulates stress-induced changes of catecholamines in the adrenal medulla. In bursa-intact chicks, CWI stress decreased E and NE within 5 minutes. In bursectomized chicks, CWI stress decreased adrenal NE within 15 minutes and E in 30 minutes. Further, it is found that E

specifically causes size reduction of bursa follicles in the chicken (De and Ghosh 1999).

Concluding remarks

During the last two decades, research from this laboratory has enlightened different aspects of avian adrenal medulla.

Several histologic and histochemical methods have been employed to explore the cytomorphology of the chromaffin cells. Studies with electron microscopes, both TEM and SEM, have also been carried out to understand the ultrastructure of the E- and NE-containing cells of a number of avian species. Investigation points out that in the normal chromaffin cells of most birds there exists specific E- and NE-storing cells. Furthermore, E/NE ratio in birds seems to bear a distinct relation to the suggested avian phylogeny as described by Gregory (1957). The latter is based on palaeontological evidences of avian phylogeny. As per recent cladistic and molecular analyses, however, the hormonal taxa of avians needs to be re-examined.

Biochemical analyses have established that along with the soluble proteins, phosphatidyl ethanolamine, lecithin, and sphingomyelin exist in the chromaffin vesicles and they play a role in the secretory process of chromaffin cells. However, it has been shown that avian adrenal phospholipid content is not dependent on the relative concentration of E and NE.

Investigation has also enlightened the release mechanism of catechol hormones from the avian adrenal medulla. Experimental application of lithium with MAO-A and MAO-B isozyme inhibitors in pigeons demonstrates a significant rise in medullary catecholamine level after blockade of type B activity. This shows a difference in the mechanism of action of MAO type A and type B in the release of catecholamines. Recent findings in pigeons leads to the inference that exocytosis may not be the only mechanism of depletion of secretary vesicles in birds. The intricate phenomenon of avian catecholamine release requires serious attention.

The neural regulation of catecholamine release from the adrenal gland has been established in this laboratory through splanchnic denervation and other experimental manipulations. Studies point out that the splanchnic nerve modulates the synthesis, release, and resynthesis of catecholamines from chromaffin cells. Moreover, the splanchnic nerve works in synergy with glucocorticoids to regulate the catecholamine content of the adrenal medulla and plays a role in controlling blood sugar levels in insulin-induced hypoglycemic birds.

Research from this laboratory has given information on the influence of catecholamines on carbohydrate and fat metabolism in birds. These studies have stressed the involvement of endocrine glands with adrenomedullary functions in birds. The influence of melatonin in modulating E and NE contents of the adrenal gland deserves a special mention. A comparative study on the effect of insulin on adrenomedullary catecholamine contents shows, that excepting the babbler and parakeet, its action on the avian adrenal medulla follows the mammalian pattern. Avian vasopressin also affects the avian medulla in a similar manner as seen in mammals.

The involvement of the avian adrenal medulla to counteract the various stress effects has been emphasized in this article. The role of vitamin A in ameliorating the stress effect in pigeons is also indicated.

In spite of an extensive research on the avian adrenal medulla, a major gap remains on the understanding of the storage and release mechanisms of catecholamines from chromaffin cells. Exploration of this field will definitely open new frontiers in avian endocrinology.

Acknowledgments

The work was supported by Indian National Science Academy (BS/SS/73/95) to A.G. and Principal, Lady Braboume College to M.M. The senior author is grateful to all his

Table 1. Effect of PG inhibitors on adrenomedullary responses in formalin stressed domestic pigeon (*Columba livia*).

Treatment	N	Epinephrine (μg/mg tissue)	Norepinephrine (μg/mg tissue)
None	(6)	0.352±0.049*	0.121±0.024
Formalin	(6)	0.227±0.029 P<0.05	0.091±0.004 NS
Apririn	(7)	0.317±0.045 NS	0.089±0.005 NS
Apririn + Formalin	(7)	0.218±0.028 P<0.05 NS**	0.071±0.004 NS NS**
Mefenamic acid	(6)	0.181±0.042 P<0.025	0.079±0.012 NS
Mefenamic acid + Formalin Diclofenac	(6)	0.079±0.019 P<0.001 P<0.005**	0.049±0.008 P<0.001 P<0.001**
Diclofenac + Formalin Piroxicam	(6)	0.461±0.040 NS	0.140±0.026 NS
Piroxicam + Formalin	(6)	0.237±0.21 P<0.050 NS**	0.084±0.006 NS NS**
	(6)	0.273±0.050 NS	0.124±0.009 NS
	(6)	0.210±0.025 P<0.025 NS**	0.104±0.005 NS NS**

* Mean ± SEM
** Formalin vs. PG Inhibitor + Formalin
Figures in parenthesis represent the number of specimens.
NS= non significant

collaborators, past and present, for their unforgettable assistance.

References

Banerjee D, Carmichael SW, Mondal A, Ghosh A (1994) Effect of water deprivation on the adrenal medulla of three Passerine birds living in different habitats. Proc Indian Natn Sci Acad B-60:9-14.

Bhattacharyya S (1971) Thyroid-adrenomedullary catechol hormone interrelationship in the avian physiology. Ph.D. thesis, 1-107, University of Calcutta.

Bobek S, Sechman A, Wieczorek E, Wronska-Frotuna D, Koziec K, Niezgoda J (1996) Responses of heat stressed chickens to exogenous reverse triiodo thyronine (rT_3)- Zentralbl Veterinarmed A 43:521-30.

Carmichael SW, Banerjee D, Mondal A, Ghosh A (1983) The effect of MAO inhibitor on reserpine-induced secretion of catecholamines from the adrenal medulla of the rose-ringed parakeet. Cell Tissue Res 22:309-316.

Carmichael SW, Banerjee D, Mondal A, Ghosh A (1985) Electron microscopical studies of the adrenal medulla of a migratory snipe, *Capella gallinago*, Charadriformes. Mikroskopie (Wien) 42:287-291.

Carmichael SW, Banerjee D, Mondal A, Ghosh A (1987) Effect of insulin on adrenal medulla in two avian species. Z Microsk Anat Forsch Leipzig 101:561-566.

Chakravorty K, Sharma KK, Bhatt D, Chandola A (1985) Control of seasonal reproduction in tropical weaver bird. In B.K. Follett, S. Ishii, Chandola A, ed., Japan Sci Soc Press, Tokyo/Springer-Verlag, Berlin, 157-165.

Chatterjee S, Ghosh A (1982) Influence of experimental scurvy on adrenomedullary catecholamine in mammals and birds. Cell Mol Biol 28:401-403.

Chattopadhyay M (1986) Adrenomedullary participation in certain aspects of avian carbohydrate metabolism. Ph.D. Thesis, University of Calcutta.

Chakrabarti E, Sengupta S, Ghosh A (1996) A comparative study of adrenal phospholipid functions in avian species. Proc Indian Natn Sci Acad B-62:383-388.

Chakrabarti E, Ghosh S, Sengupta S, Ghosh A (1999) Adrenal phospholipid profile during lithium treatment in domestic pigeon. Ind J Physiol and Allied Sci 53:91-95.

Chaudhuri D, Ghosh I, Ghosh A (1966) Steroidal influence on adrenomedullary catechol hormones of the pigeon. Acta Morphol Acad Sci Hung 14:245-252.

Chungsmarnyart N, Fujioka T (1982) Ultrastructural studies on the degranulation and regranulation of adrenal catecholamine storing cells in the domestic fowl after reserpine treatment. Jpn J Vet Sci 44:439-457.

Ciaranello RD (1978) Regulation of phenylethanolamine *N*-methyl transferase synthesis and degradation. 1. Regulation by rat adrenal glucocorticoids. Mol Pharmacol 14:478-489.

Coupland RE (1971) In Subcellular Organization and Function in Endocrine Tissues. In Heller H, Lederis K, eds., Cambridge Univ Press, London. 611-635.

Cracraft J (1988) The phylogeny and classification of the tetrapods: Amphibians, reptiles, birds. In The major glands of birds. Benton MJ, ed., Systemat Ass Special Vol 35A, Clarendon Press Oxford. 339-362.

Cuello AC (1970) Occurrence of adrenaline and noradrenaline cells in the adrenal gland of the gentoo penguin (*Pygoscelis papua*). Experientia 26:416-418.

Das Adhikari (Agarwal), Guha S and B (1991) Effect of formalin stress on the adrenal functions of the domestic pigeon (*Columba livia*). J Yamashina Inst Ornithol 23:107-110.

De K, Ghosh A (1993) Influence of dehydration stress on the adrenal glands of two semi-domesticated avian species. J Yamashina Inst Ornithol 25:68-75.

De M, Ghosh A (1999) Effect of catecholamines on the bursa of pigeon. Ind J Exp Biol 37:311-313.

Diner 0 (1967) L'expulsion des vesicles de la Medullosurrenale chez le Hamster. C R Acad Sci 265:616.

Ghosh A (1962) A comparative study of the histochemistry of the avian adrenals. In Progress in Comparative Endocrinology. Takewaki K, ed., 75-80. Academic Press, New York.

Ghosh A (1977) Cytophysiology of the avian adrenal medulla. Int Rev Cytol, Bourne G, Danielli JP, eds., 253-284. Academic Press New York.

Ghosh A (1980) Structure and functions of avian adrenal medulla. In Avian Endocrinology, 301-308. Academic Press, New York.

Ghosh A, Sitaraman S (1975) Effect of dehydration on the adrenal gland of the pigeon. Proc Symp Temp Regulation, 1122-1124.

Ghosh A, Chatterjee S (1986) In Current trends in comparative Endocrinology. Lofts B, Holmes WN, eds., Hong Kong Univ Press, Hong Kong, 1013-1015.

Ghosh A, Chatterjee S (1986) The avian adrenal medulla and the effect of ascorbic acid on adrenomedullary hormones. Indian Rev Life Sci 6:67-73.

Ghosh A , Guha B (1988) The subcellular morphology of avian adrenal medulla. Proc Ind Acad Sci (animal science) 97:329-337.

Ghosh S, Guha B, Das Adhikari (Agarwal) S, Sengupta S (1992) Can Vitamin A ameliorate chemical and psycho-physical stressors in domestic pigeon? An experimental study. Proc Zool Soc Calcutta 45 (Suppl A): 33-38.

Ghosh, S, Carmichael SW, Banerjee D, Mondal A, Ghosh A (1993) Effect of water deprivation on the adrenal medulla of three passerine species living in different habitats. J Electron Microsc Soc Thailand 7:60.

Ghosh S, Sengupta S, Das Adhikari S, Chakrabarti E, Sarkar S, Ghosh A (1995) The role of monoamine oxidase isozymes on guanethidine induced adrenal medullary catecholamine release in *Columba livia*. Cytobios 83:249-255.

Ghosh S, Carmichael SW, Guha B, Banerjee D, Ghosh A (1996a) Comparative ultrastructural study of the adrenal medulla in some subtropical avian species. The Nucleus 39:90-106.

Ghosh S, Sengupta S, Das Adhikari S, Ghosh A (1996b) Relation between the adrenals and thyroid and their hormones and the testicular cycle of a subtropical avian species. Biol Rhythm Res 27:216-226.

Ghosh S, Sengupta S, Ghosh A (1997a) Effect of chemical adrenomedullectomy on the testicular physiology of pigeons. Cytobios 90:163-170.

Ghosh S, Sengupta S, Sarkar S, Ghosh A (1997b) The role of MAO isozymes on pigeon adrenal medulla during lithium treatment. Biomed Res 8:53-56.

Ghosh S, Sengupta S, Chakraborty M, Ghosh A (1999a) Action of lithium on chromaffin cells of the domestic pigeon (*Columba livia*). The Nucleus 42:28-33.

Ghosh S, Sengupta S, Ghosh A (1999b) Effect of lithium in modulation of adrenal hormones in pigeon during hyperthyroid condition. J Animal Health 38:27-29.

Goebel S, Diebrich M, Jarry H, Waltke W (1992) Indirect evidence to suggest that prolactin mediates the adrenal action of haloperidol to stimulate aldosterone and corticosterone secretions in rat. Endocrinol 130:914-919.

Gregory W K (1957) "Evolution Emerging" Vol. II .McMillan, New York.

Guha B, Pal D, Carmichael SW, Ghosh A (1990) Morphology of the avian adrenal gland examined by scanning electron microscopy. Z Microsk Anat Forsch 104:395-400.

Guha B, Pal D, Ghosh A (1992) An immunocytochemical study of phenylethanolamine *N*-methyltransferase (PNMT) in the adrenal medulla of four birds. Nat Acad Sci Lett 15:333-335.

Guzsal E, Hassan A (1975) Ultrastructure of adrenal medullary cells in the goose and duck. Acta Vet Acad Sci Hung 25:321-333.

Khalil Z, Livett BG, Marley PD (1986a) The role of sensory fibres in the rat splanchnic nerve in the regulation of adrenal medullary secretion during stress. J Physiol (London) 370:201-215.

Khalil Z, Livett, BG, Marley PD (1986b) Elevation of plasma catecholamines in response to insulin-stress under both neuronal and non-

neuronal control. Endocrinol 119:159-167.
Khalil Z, Livett BG, Marley PD (1987) Sensory fibres modulate histamine-induced catecholamine secretary from the rat adrenal medulla and sympathetic nerves. J Physiol (London) 391:511-526.
Kach T, Banerjee TK, Quay WB (1988) Quantitative cytological analysis of functional changes in adrenomedullary chromaffin cells in normal, sham-operated and pinealectomized rats in relation to time-of-day. J Pineal Res 5:527-534.
Kobayashi H, Uemura I, Takei Y (1980) Physiological role of the renin angiotensin system during dehydration. Avian Endocrinol. In Epple A, Stetson MH, eds., Academic Press, New York. 319-330.
Ljunggren L (1969) Seasonal studies of wood pigeon populations. II. Gonads, crop glands, adrenals and the hypothalamo-hypophysial system. Viltrevy 6:41-126.
Lucas CA, Thoenen H (1977) Selective induction by glucocorticoids of tyrosine hydroxylase in organ cultures of rat pheochromocytoma. Neurosci 2:1095-1101.
Mahata SK, Ghosh A (1986a) Influence of splanchnic nerve on the resynthesis of adrenomedullary catecholamines post-reserpine-induced depletion in the pigeon, *Columba livia*. Proc Indian Nat Sci Acad B-52:346-350.
Mahata SK, Ghosh A (1986b) Influence of splanchnic nerve and age on the action of insulin in the adrenomedullary catecholamine content and blood glucose level in pigeon. Arch Biol (Bruxelles) 97:443-454.
Mahata SK, Ghosh A (1986c) Influence of age on adrenomedullary catechol hormones content in twenty-five avian species. Boll Zool 53:63-67.
Mahata SK, Mandal A, Ghosh A (1988) Influence of age and splanchnic nerve on the action of melatonin in the adrenomedullary catecholamine content and blood glucose level in the avian group. J Comp Physiol B 158:601-607.
Mahata SK, Ghosh A (1989) Influence of splanchnic nerve on reserpine action in avian adrenal medulla. Gen Comp Endocrinol 73:165-172.
Mahata SK, De M, Pal D, Ghosh A (1990c) Effect of stress on the catecholamine content of the adrenal gland of intact and bursectomized chicks. Clin Exp Pharmacol Physiol 17:805-808.
Mahata SK, De M, Pal D, Ghosh A (1990a) Neural influence on the action of insulin in the adrenomedullary catecholamine content in the pigeon. Neurosci Lett 116:336-340.
Mahata SK, De M, Pal D, Ghosh A (1990b) Effect of insulin on adrenomedullary catecholamine content and blood glucose level in normal and reserpinized avian species. Biogenic Amines 7:455-464.
Mahata S, Ghosh A (1991) Neural modulation of lysine vasopressin-induced changes of catecholamines in the adrenal medulla of the pigeon. Neuropeptides, 18:29-33.
Mahata SK, De K (1991) Influence of age and splanchnic nerve on glucagon-induced changes of adrenomedullary catecholamine content and blood glucose level in the avian group. J Comp Physiol 161:81-84.
Maitra SK, Dey M (1992) Importance of photoperiods in determining temporal pattern of annual testicular events in rose-ringed parakeet (*Psittacula krameri*). J Biol Rhythms 7:13-25.
Maitra SK, Ghosh A (1980) Cytological studies of two different hormone containing cells in the adrenal medulla of the bird, *Psittacula cyanocephala*. Proc Indian Sci Cong Assoc, Part III, Abst. 115.
Maitra SK, Ghosh A (1981) Gonadal response to testosterone propionate during breeding and post-breeding phase of the male blossom headed parakeet. Aust J Zool 29:853-860.
Mallick B, Sarkar AK (1985) Studies on the seasonal cyclicity of the testis and adrenal in the common myna (*Acridotheres tristis tristis*). J Reprod Biol Comp Endocrinol 5:80-88.
Malmfors T. Thoenen H (1971) 6-Hydroxydopamine and catecholamine neurons. North Holland, Amsterdam.
Marley PD, Mitchelhill KI, Livett BG (1985) Effects of opioid peptides containing the sequence of Met^5-enkephalin or Leu^5-enkephalin on nicotine-induced secretion from bovine adrenal chromaffin cells. J Neurochem 46:1 -11.
Mindell DP (1997) Avian Molecular Evolution and Systematics. Academic Press, London.
Mukherjee M, Das Adhikari S, Ghosh A (1990. The role of prolactin on the adrenal gland of parakeet (*Psittacula krameri*): A histophysiological study. Proc Zool Soc Calcutta 43:1-7.
Mukherjee M (1985) The effect of catecholamines on the testis of weaver bird (*Ploceus philippinus*). Mikroskopie (Wien) 42:39-44.
Mukherjee M (1988) The mechanism of action of epinephrine on the testis of weaver bird (*Ploceus philippinus*). J Yamashina Inst Ornith 20:41-45.
Mukherjee M, Mondal A, Ghosh S, Pakrashi A, Ghosh A (1993) Effect of prolactin on the adrenogonadal activity of the male pigeon (*Columba livia*). Folia Biologica (Krakow) 41:33-36.
Mukherjee M, Chatterjee S, Pakrashi A, Ghosh A (1995) Adrenal gland response to a dopamine antagonist in rat. Biogenic Amines 11:333-338.
Nussdorfer GG (1996) Paracrine control of adrenal cortical function by medullary chromaffin cells. Pharmacol Rev 48:495-530.
Nussey SS, Prysol-Jones RA, Taylor AH, Ang V, Jenkins JS (1987) Arginine vasopressin and oxytocin in the bovine adrenal gland. J Endocrinol 115:141-14.
Ohmori Y (1998) Localization of biogenic amines and neuropeptides in adrenal medullary cells of birds. Horm Metab Res 30:384-388.
Ohmori Y, Okada Y, Watanabe T (1997) Immunohistochemical localization of serotonin, galanin, cholecystokinin and methionine-enkephalin in adrenal medullary cells of the chicken. Tissue Cell 29:199-205.
Olson JA (1984) Handbook of Vitamins. Marcel Dekker Inc., New York.
Pal D, Chatterjee S (1988) Influence of testosterone on adrenomedullary catecholamines during corticoid inhibition in pigeon. Proc Zool Soc Calcutta 38:1-3.
Pilo B, John TN, Pemasingh RS, George JC (1984) Cytobios 41:175-180 [vide R.J. Verma and B. Pilo (1987)].
Pohorecky LA, Wurtman RJ (1971) Adrenocortical control of epinephrine synthesis. Pharmacol Rev 23:1-35.
Porter ID, Whitehouse BJ, Taylor AH, Nussey SS (1988) Effect of arginine vasopressin and oxytocin on acetylcholine stimulation of corticosteroid and catecholamine secretion from the rat adrenal gland perfused in situ. Neuropeptides 12:265-271.
Rogers CA, Cécyre D, Lemaire S (1989) Presence of delta and phencyclidine (PCP)-like receptors in membrane preparations of bovine adrenal medulla. Biochem Pharmacol 38:2467-2472.
Role LW, Perlman RL (1983) Both nicotinic and muscarinic receptors mediate catecholamine secretion by isolated guinea pig chromaffin cells. Neurosci 10:979-985.
Ross S, Fischer A, Unsicker K (1995) Sympathoadrenal progenitors in embryonic chick sympathetic ganglia show distinct responses to glucocorticoid hormones. J Neurocytol 24:247-256.
Sanchez-Montesinos I, Merida-Velasco JA, Espin-Ferra J, Scopsi L (1996) Development of the sympathoadrenal system in the chick embryo: An immunocytochemical study with the antibodies to pan-neuroendocrine markers, catecholamine synthesizing enzymes, proprotein-processing enzymes and neuro- peptides. Anat Rec 245:94-101.
Sarka S, Sengupta S, Das, Adhikari S, Ghosh S, Ghosh A (1996) Effect of ibuprofen, a prostaglandin blocker, on the adrenal hormones of the pigeon during stress. Nat Acad Sci Lett 19:129-131.
Sarkar S, Ghosh S, Sengupta S, Dasadhikari S, Ghosh A (1999) Action of chemically different prostaglandin blockers on the adrenal hormones during stress in pigeons. Ind J Physiol Pharmacol 43:84-88.
Sen D, Bhattacharya SP (1984) Fasting induced alterations in plasma FFA, blood sugar and glycogen in the rose-ringed parakeet and collard scops owl. Comp Physiol Ecol 9:155-158.
Sengupta S, Das Adhikari S, Guha B, Ghosh S, Ghosh A (1994) The role of prostaglandin in the modulation of adrenal catechol hormones and corticosterone in the pigeon during stress. Biogenic Amines 10:561-564.
Sitaraman S, Ghosh A (1979) The role of corticomedullary hormones in avians exposed to stress stimuli. Biol Bull Ind 1:33-37.
Sturkie PD (1965) Avian Physiology. Bailliere, London.

Subramanian N (1980) Mini review on the brain ascorbic acid and its importance in metabolism of biogenic amines. Life Sci 20:1479-1484.

Takahashi JS, Hamm H, Menaker M (1980) Circadian rhythms of melatonin release from individual superfused chicken pineal glands in vitro. Proc Natl Acad Sci USA 77:2319-2322.

Unsicker K (1973a) Fine structure and innervation of the avian adrenal gland. Fine structure of adrenal chromaffin cells and ganglion cells. Z Zellforsch 145:389-416.

Unsicker K (1973b). Fine structure and innervation of avian adrenal gland. II Cholinergic innervation of adrenal chromaffin cells. Z Zellforsch 145:417-442.

Unsicker K (1973c). Fine structure and innervation of avian adrenal gland. III Non- cholinergic nerve fibres. Z Zellforsch 145:557-575.

Vestergaard K, Willeberg P (1978) Video scanning for determination of the proportion of corticol tissue in the avian adrenal gland. Acta Vet Scand 19:331-340.

Vitale N, Deloulme J-C, Thiersé D, Aunis D, Bader M-F (1994) GAP-43 controls the availability of secretory chromaffin granules for regulated exocytosis by stimulating a granule-associated G_O. J Biol Chem 269:30293-30298.

Wakade AR, Wakade TD (1983) Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and erogenous acetylcholine. Neurosci 10:973-978.

Wasserman GF, Bernard EA (1971) The influence of corticoids on the phenylethanolamine-*N*-methyl transferase activity in the adrenal gland of Gallus domesticus. Gen Comp Endocrinol 17:83-93.

Wood JG (1963) Identification of and observations of epinephrine and norepinephrine containing cells in the adrenal medulla. Am J Anat 112:285-303.

Wurtman RJ, Axelrod J (1965) Adrenaline synthesis: Control by the pituitary gland and adrenal glucocorticoids. Science 150:1464-1465.

Wurtman RJ (1966) Control of epinephrine synthesis in the adrenal medulla by the adrenal cortex: Hormonal specificity and dose-response characteristics. Endocrinol 79:392-394.

Glossary of abbreviations

TH	tyrosine hydroxylase (EC 1.14.16.2)
CWI	cold-wet immobilization stress
DbH	dopamine β-hydroxylase (EC 1.14.17.1)
E	epinephrine
FFA	free fatty acids
LiCl	lithium chloride
NE	norepinephrine
NPY	neuropeptide Y
PNMT	phenylethanolamine *N*-methyltransferase (EC 2.1.1.28)
TEM	transmission electron microscopy
SEM	scanning electron microscopy

Volume 45(1-4):13-21, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

REVIEW ARTICLE

Heterotrimeric G-proteins and their role in opioid receptor function

Gabriella Fábián

Department of Zoology and Cell Biology, University of Szeged, Szeged, Hungary

ABSTRACT Heterotrimeric G-proteins are signal transducers of heptahelical receptors. They consist of α and βγ subunits, both capable of interacting with several different effectors. Specific domains in their structures enable them to connect different intracellular signaling cascades, such as the adenylyl cyclase, phosphoinositol-bisphosphate or MAP kinase pathways. Their activity is synchronized by several components, one of them being a new protein family termed RGS (regulators of G-protein signaling). Members of this family inhibit the G-protein function. The intracellular localization of G-proteins indicates their role in plasma membrane-independent processes. Opioid receptors transmit their signals mainly via $G_{i/o}$ proteins. Although the heterogeneity of opioid ligands (peptides and alkaloids) and their receptors (μ, δ, κ and suggested subtypes in these classes) reveals a complicated picture, their unique characteristic of a high dependence capacity can not be explained without the analysis of the G-protein function.
Acta Biol Szeged 45(1-4):13-21 (2001)

KEY WORDS

signal transduction
G-proteins
opioid receptor
tolerance

G-proteins

General features of structure and function

The heterotrimeric guanine nucleotide binding proteins (G-proteins) have been discovered about 20 years ago, and the key nature of their participation in signal transduction led to their discoverers being honored with the Nobel Prize for medicine in 1994. They function as intermediaries in transmembrane signaling pathways that involve three proteins: receptors, G-proteins, and effectors (Gilman 1987). They belong in the superfamily of GTPases, which includes factors that take part in protein synthesis (*e.g.* elongation factor Tu) and small molecular weight (20-25 kDa) monomeric G-proteins, such as p21 ras and its relatives (Hall 1990; Bourne et al. 1990; Bourne et al. 1991; Kaziro et al. 1991). G-proteins consist of three subunits, designated α, β and γ. Traditionally, the type of the α subunit is used to define the G-protein oligomer. To date, 23 distinct α subunits encoded by 17 genes have been cloned with molecular masses between 39 and 46 kDa (Gudermann et al. 1997). They can be divided into four subfamilies, G_s, G_i, G_q and G_{12}, based on amino acid sequence homology. Some of them are ubiquitous, *e.g.* α_s, while others are more or less specialized, for example, α_o for brain tissue or α_{t1} and α_{t2} for retinal rods and cones, respectively. G-protein α subunits are enzymes with inherent GTPase activity. They are also subject to several cotranslational and posttranslational modifications. α_i, α_o and α_z are myristoylated at their N-terminus (Mumby et al. 1990); others are modified by different saturated or non-saturated 12- and 14-carbon fatty acids, facilitating the membrane attachment of α subunits and increasing their affinity for βγ dimers (Linder et al. 1991). In addition to this irreversible lipid modification, some α subunits, *e.g.* α_s, are reversibly palmitoylated on the cysteine residue nearest the amino terminus, which seems to have a regulatory function (Wedegaertner and Bourne 1994). While irreversible modifications are usually observed in the endoplasmic reticulum, this reversible lipidation occurs in the cytoplasm. Upon receptor activation, $G_s\alpha$ undergoes substantial depalmitoylation, which may be further increased by cholera toxin. Inactivation of the $G_s\alpha$ subunit is associated with repalmitoylation, which inhibits the interaction of this subunit with other regulatory proteins, *e.g.* Gα-interacting protein (GAIP). The lipid sensitivity of the G-protein function implies also that the lipid composition of the membrane microdomains can influence the signaling (Green et al. 1999). A characteristic modification of certain types of G-protein α subunits is the ADP-ribosylation by bacterial toxins. Pertussis toxin catalyzes the covalent binding of ADP-ribose to a cysteine residue located four amino acids from the C-terminus. All α_o and α_i subunits can be modified in this way, resulting in uncoupling from the receptor by inhibiting the activation of the α subunit. Cholera toxin specifically ADP-ribosylates an arginine residue in α_t, α_s and α_{olf}, leading to inhibited GTPase activity, that hence to constitutive activation of those α subunits (Hepler and Gilman 1992). There are also several possible sites for phosphorylation.

Accepted May 18, 2001
E-mail: fabiang@bio.u-szeged.hu

Five β (35-37 kDa) and 12 γ (8 kDa) subunits have been described to date (Watson et al. 1994; Ray et al. 1995; Morishita et al. 1995). They are tightly associated and form one functional unit. There is evidence that a degree of specificity governs βγ dimer assembly, and not all possible combinations are formed (reviewed in Gudermann et al. 1997). Gamma subunits are either farnesylated or geranylgeranylated, which furnishes the anchorage to the plasma membrane. It is generally considered that the β subunit interacts with the α subunit, while the γ subunit determines the effector specificity in the action of the dimer.

Role of G-proteins in signal transduction

Receptor-G-protein interaction

G-proteins serve as membrane-bound transducers of chemically and physically coded information. This extracellular information is received by receptor (R) molecules that are integrated plasma membrane proteins. Certain classes of such receptors (*e.g.* ligand-gated ion channels or tyrosine kinase receptors) themselves have effector domains, whereas others, characterized by 7 transmembrane α helical domains (7TM receptors or G-protein-coupled receptors, GPCRs), first activate G-proteins, which in turn activate the effector molecules. The steps in this cycle are presented in Fig. 1.

It is usually the third intracellular domain and the C-terminal intracellular tail of the receptor molecule that determine the R-G-protein interaction. For the activation of G-proteins, Mg^{2+} and GTP are essential. Little is known about the regulation of the GTPase cycle, since it proceeds 10 to 100 times faster *in vivo* than *in vitro*. However, several proteins with GTPase-activating properties (GAPs) for Gα subunits were recently described. They are termed regulators for G-protein signaling (RGS; Watson et al. 1996). At least 20 different mammalian proteins have been reported to have an RGS core, a common 120 amino acid domain. Although the number of different Gα subunits is close to this, there is not a one to one correspondence between them, and no RGS specific for $G_s\alpha$ and $G_{12}\alpha$ has so far been identified. The GTPase-activating domain acts catalytically: a single molecule of RGS can accelerate the GTPase activity of 4-6 Gα subunits. They not only provide enhancement of the enzymatic activity for most of the Gα subunits, but may also function as effector antagonists and integrators of different signaling pathways, in consequence of their C- and N-terminal protein binding motifs (Burchett 2000). One of them is the GGL (G-protein gamma subunit-like) domain, which, *e.g.* in human RGS11, has been shown to form a complex with Gβ5 (Snow et al. 1998). The RGS11/Gβ5 complex is a selective regulator of $G_o\alpha$.

G-proteins are also signal amplifiers. This can be achieved at different levels. First, a single receptor can activate several G-proteins in turn; second, the dissociation of α and βγ subunits leads to bifurcation of the signal; and on the third level, G-protein subunits can activate several effector molecules before reassociation (Milligan 1996).

G-protein-effector interaction

Recent results show that, upon activation of a G-protein, both α and βγ subunits are able to interact with different effectors (Birnbaumer 1992) to induce further changes in the state of the cell, leading to a response to the extracellular stimulus, or, in a broader sense, to adaptation. The effectors and their activator G-protein subunits are listed in Tables 1 and 2.

Influence of G-proteins on the gene expression

One main pathway for the regulation of gene expression by extracellular signals transduced by GPCRs proceeds via the activation of adenylyl cyclase and the subsequent production of cyclic AMP (cAMP). cAMP regulates the transcription of a variety of genes through a distinct DNA sequence termed the cAMP response element (CRE), present in their promoter regions. This element is recognized by the CRE-binding protein (CREB), a transcription factor of 43 kDa. Activation of CREB is achieved by cAMP-dependent protein kinase

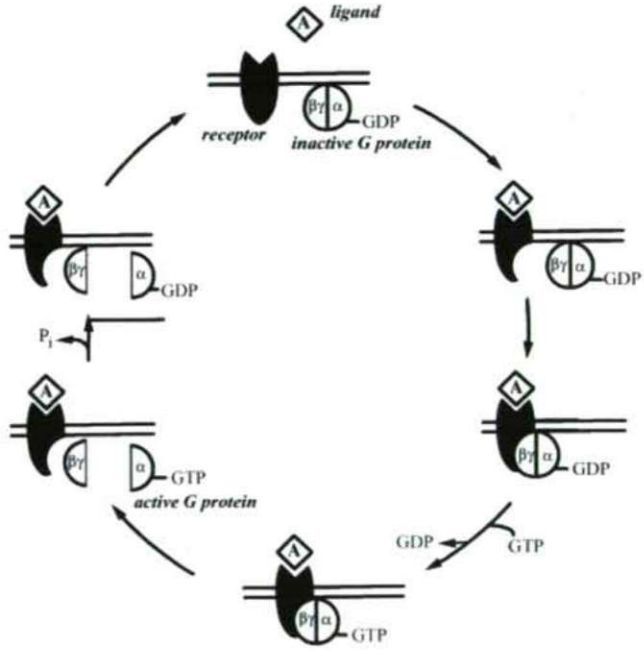


Figure 1. Ligand-activated GTPase cycle of G-proteins. In the resting state, heterotrimeric G-proteins bind GDP. The ligand-bound receptor can activate the G-protein resulting in the exchange of GDP by GTP and subsequent dissociation of α-GTP and the βγ dimer, each of them capable of activating effectors. The effect is terminated by the inherent GTPase activity of the α subunit and the reassociation of α-GDP with βγ. R: receptor, A: agonist ligand.

(PKA; Goodman 1990; Montminy et al. 1990; Collins et al. 1992; Zazopoulos et al. 1997)

The other pathway by which G-proteins can exert an influence is the signaling route of the receptor tyrosine kinases, such as epidermal growth factor, leading to cell differentiation, proliferation and cytoskeletal effects through the mitogen-activated protein kinase (MAPK) cascade. There are several convergence points between the two signal transduction pathways (for reviews, see Selbie and Hill 1998; Seasholtz et al. 1999; Pierce et al. 2001).

Role of intracellular G-proteins

Heterotrimeric G-proteins are found not only in the plasma membrane fractions, but also inside the cell, in the cytoplasm or connected to the endomembrane systems such as the Golgi and the endoplasmic reticulum. They can be detected in the non-nervous tissues, such as the liver (Lanoix et al. 1989; Toki et al. 1989), in the muscle (Carrasco et al. 1994) and also in the brain (Bem et al. 1991; Holz and Tutner 1998).

These intracellular G-proteins can be newly synthesized molecules, which are transported to the cell surface, probably in a fully functional state, able to interact with receptors and also with effectors (Zarbin et al. 1990; Vogel et al. 1991). Intracellular G-proteins may also be conveyed from the cell surface as part of the signal transduction process (Zarbin et al. 1983; Laduron 1992; Szűcs and Coscia 1992). Several plasma membrane receptors have a nuclear localization signal in their cytoplasmic tail; accordingly, they, or part of them, can enter the nucleus either alone or with other proteins recruited during the signaling process (Laduron 1994). However, recent results have revealed that G-proteins are not only transported as passive molecules, but they also have important intracellular functions. They have been suggested to regulate various membrane trafficking processes, including several steps of secretion. Coat assembly and the sorting of newly synthesized proteins secreted constitutively in polarized cells appear to be controlled by heterotrimer G-proteins (Ktistakis et al. 1992; Robinson and Kreis 1992; Pimplikar and Simons 1993). The processes of exocytotic and endocytotic membrane fusion are also under the stimulatory control of G_i and the inhibitory control of G_o (Bomsel and Mostov 1992; Ahnert-Hilger et al. 1994; Colombo et al. 1994; Helms 1995). A role of G-proteins in the maintenance of the highly specialized structure of the blood-brain barrier has also been suggested (Brett et al. 1989; Hoyer et al. 1991; Raub 1996; Fábián et al. 1998).

The opioid receptors

Opioid receptor types and function

Opioid receptors also belong in the family of GPCRs, and are characterized by 7 hydrophobic transmembrane segments and the ability to interact with different G-proteins (McKenzie and Milligan 1990; Offermanns et al. 1991; Laugwitz et al. 1993). Opioid receptors have been identified in pharmacological studies through the use of peptide and alkaloid ligands, and have been classified into three main classes, μ, δ and κ (Martin et al. 1976). Cloning of the receptors has verified this model (Kieffer et al. 1992; Evans et al. 1992; Chen et al. 1993; Yasuda et al. 1993), but failed to prove the

Table 1. Mammalian G-protein a subunits and effectors interacting with them

Subtype	Expression	Effectors
α_{s} (2 forms)*	Ubiquitous	Adenylyl cyclase ↑ (all types)
α_{xL} (2 forms)*	Ubiquitous	Ca^{2+} channel ↑ (L-type)
α_{olf}	Olfactory epithelium	Adenylyl cyclase ↑ (type V)
α_{gust}	Taste buds, gut	?
α_{t-r}	Retinal rods	cGMP phosphodiesterase ↑
α_{t-c}	Retinal cones	
α_{i1}	Widely	Adenylyl cyclase ↓
α_{i2}	Ubiquitous	(types I, III, V, VI)
α_{i3}	Nearly ubiquitous	K^+ channel ↑
α_{o1}*	Neuronal and neuroendocrine	Ca^{2+} channels ↓
α_{o2}*	Neuronal and neuroendocrine	(Types L and N)
α_{z}	Neuronal, platelets	Adenylyl cyclase ↓ ?
α_{q}	Ubiquitous	Phospholipase-Cβ ↑
α_{11}	Ubiquitous	(β4 ≥ β1 ≥ β3 > β2)
α_{14}	Kidney, lung, spleen	
α_{15} (mouse)	Hematopoetic cells	
α_{16} (human)		
α_{12}	Ubiquitous	?
α_{13}	Ubiquitous	?

G-protein a subunits form 4 families based on sequence homology. * Splice variants. The data was taken from Weiland et al. (1997).

existence of the opioid receptor subtypes proposed for all three classes on the basis of pharmacological studies. This suggests that the pharmacological subtypes may result from posttranslational splicing modifications or differential protein-protein interactions between receptors or with associated proteins. The gene structures of all three opioid receptors afford the possibility of alternative splicing, and different mRNA products of single opioid receptor gene have indeed been detected (Gaveriaux-Ruff et al. 1997; Schulz et al. 1998). It has also been demonstrated that proteins encoded by the mRNA isoforms of the μ opioid receptor are desensitized at different rates (Koch et al. 1998). Extensive evidence of pharmacological and functional interactions between opioid receptor types has accumulated (reviewed by Jordan et al. 2000). These studies show that heterodimers, such as μ/δ, exhibit distinct ligand binding and signaling characteristics. Additional signaling features and regulation occur when δ or κ opioid receptors form heterodimers with β_2-adrenergic receptors (Jordan et al. 2001). This heterooligomerization does not alter the ligand binding or coupling properties of the receptors (although they couple to different classes of G-proteins, *i.e.* $G_{i/o}$ and G_s), but it affects their trafficking. When coexpressed with β_2 receptors, δ opioid receptors undergo isoproterenol-mediated endocytosis. Conversely, the β_2 receptors in these cells undergo etorphine-mediated endocytosis. However, the coexpression of κ opioid receptors with β_2 receptors blocks opioid- and isoproterenol-mediated endocytosis. Ligand-specific regulation of the endocytosis has been detected for homooligomers of the δ receptor. Homodimer formation is reduced by increasing concentrations of agonists, such as DADLE, DPDPE or etorphine, while morphine is ineffective. Depolimerization of the dimers correlated with the internalization of the receptor (Cvejic and Devi 1997).

Pharmacological effects of the opioid receptors are listed in Table 3.

Ligand binding to the opioid receptor

Radioligand binding studies combined with site-directed mutagenesis of the receptor molecules have provided a great deal of information on the interactions of opioid ligands with their receptors (for a review, see Raynor et al. 1996). It is thought that only agonist binding leads to activation of the receptor, followed by conformational changes and information transfer. In contrast, antagonist binding does not elicit a biological response. Certain charged amino acids in the transmembrane regions TM II (Asp114), III (Asp147) and VI (His297) have been shown to be important for ligand binding and subsequent activation of effectors (Surrat et al. 1994). Further, opioid peptides and alkaloids, and also agonists and antagonists bind to different parts of the receptor molecule (Zastrov et al. 1993; Surrat et al. 1994). In the case of δ receptors, the third extracellular loop is likewise important for ligand selectivity (Quock et al. 1999). Identification of the specific residues in the κ receptor involved in agonist and antagonist binding may facilitate the further development of therapeutically useful opioids since κ agonists have minimal abuse potential and do not cause respiratory depression, two major side-effects of the use of μ receptor-selective agonists. Nonetheless, κ agonists are effective analgesics and useful diuretic agents. Previous results have revealed that frog (*Rana esculenta*) brain membranes are suitable for the investigation of this opioid receptor type, since they contain a high proportion of κ receptors as compared to μ and δ receptors

Table 2. Mammalian G-protein b and g subunits and effectors interacting with them

Subtype	Expression	Effectors
β_1	Ubiquitous	Adenylyl cyclase ↓ (type I)
β_2	Ubiquitous	Adenylyl cyclase ↑ (types II, IV)
β_3	Ubiquitous	Phospholipase-Cβ ↑
β_4	Ubiquitous	(β3 ≥ β2 ≥ β1 > β4)
β_{5S}*	Mainly brain	K^+ channel ↑
β_{5L}*		Retina Ca^{2+} channels ↓
		Receptor kinases (types 2, 3) ↑
		Phospholipase-A_2 ↑ ?
γ_1*	Retinal rods	Phosphoinositide 3-kinase ↑ ?
γ_2	Mainly brain	?
γ_3	Mainly brain	?
γ_4	Mainly brain	?
γ_5	Ubiquitous	?
γ_7	Widely distributed	?
γ_8*	Retinal cones	?
γ_{10}	Widely distibuted	?
γ_{11}*	Widely distributed	?
γ_{12}	Ubiquitous	?

βγ combinations apparently not formed are $\beta_2\gamma_1$ and $\beta_2\gamma_{11}$; tissue-specific combinations are $\beta_1\gamma_1$ for retinal rods and $\beta_3\gamma_8$ for retinal cones. * Splice variants. • These γ subunits are farnesylated; all others are geranylgeranylated. The data was taken from Weiland et al. (1997).

(Simon et al. 1984). Frog brain membranes also contain κ receptor subtypes, *i.e.* κ_1 and κ_2 (Benyhe et al. 1990; Wollemann et al. 1993). A detailed characterization of these binding sites in ligand binding studies indicated that they might couple to G-proteins (Benyhe et al. 1991; Rottmann et al. 1994; Bozó et al. 2000).

Another mode of investigation of ligand-receptor interactions considers energetic aspects. Thermodynamic analysis provides a means of determining the underlying driving forces of binding and intermolecular interactions; such information can not be easily obtained by other techniques. Thus, conformational changes or protein-protein associations should provoke characteristic thermodynamic behavior. With this approach, it has been established that opioid agonist binding is mainly entropy driven, while opioid antagonist binding is exothermic, and therefore enthalpy driven (Nicolas et al. 1982; Hintzemann et al. 1985; Zeman et al. 1987; Borea et al. 1988; Fábián et al. 1996).

Opioid binding is modulated by a number of reagents. Na^+ and GTP decrease agonist binding without affecting antagonist binding. Divalent cations also differentiate agonist and antagonist binding (Szűcs et al. 1987; Benyhe et al. 1989 and references therein). These agents are also to be required for the functional coupling of opioid receptors to inhibitory G-proteins (Blume et al. 1979; Childers 1991; Johansson et al. 1992). By means of thermodynamic investigations, additional information can be expected about this signal transduction step. Na^+ or Mg^{2+} results only in quantitative changes in the thermodynamic parameters. In the presence of the GTP analog Gpp(NH)p, or Gpp(NH)p + Na^+ or Gpp(NH)p + Na^+ + Mg^{2+}, the affinity of dihydromorphine binding decreases dramatically, which might reflect functional uncoupling of the receptor-ligand complex and G-proteins. These altered molecular interactions are also indicated by the curvilinear van't Hoff plot and entropy increase (Fábián et al. 1996).

Consequences of repeated ligand administration

The chronic use of opiates results in drug addiction, including tolerance to and dependence on the drug; besides its scientific importance, this phenomenon has a great social impact. Despite intensive research in this field, the precise molecular mechanism that accounts for it is unknown.

In biochemical terms, the long-term presence of the agonist generally leads first to desensitization, which means that the receptor is unable to activate effector molecules in consequence of the uncoupling of the receptor from the transducer G-protein. The reason for this is the phosphorylation of the receptor by specific kinases, such as β-

Table 3. Opioid receptor pharmacology

Receptor	Biochemical effects	Physiological effects
μ	cAMP inhibition Stimulation of IP_3 formation Ca^{2+} channel inhibition K^+ channel stimulation increase of intracellular Ca^{2+}	Analgesia Sedation Immunosuppression
μ_1		Supraspinal analgesia Prolactin release Acetylcholine turnover feeding
μ_2		Spinal analgesia GH release stimulation Respiratory depression Inhibition of GI transit GPI motility decrease
Morphine-6β-glucuronide		Inhibition of GI transit
κ		Analgesia Dysphoria
κ_1	Inhibition of cAMP accumulation Inhibition of PI hydrolysis Ca^{2+} channel inhibition K^+ channel stimulation	Spinal analgesia Diuresis, sedation Rabbit *vas deferens* bioassay
κ_2	Pharmacology unknown	Pharmacology unknown
κ_3 KOR-3/ORL-1	Inhibition of cAMP accumulation K^+ channel stimulation Inhibition of cAMP accumulation	Hyperalgesia (early) Supraspinal analgesia (later)
δ	Inhibition of cAMP accumulation K^+ channel stimulation increase of intracellular Ca^{2+}	Analgesia (spinal, supraspinal) Mouse *vas deferens* bioassay Dopamine turnover inhibition GH release stimulation
δ_1		GI motility decrease
δ_2		GI motility decrease

The data was taken from Standifer and Pasternak (1997) with modifications. GH: growth hormone, GI: gastrointestinal, GPI: guinea pig ileum.

adrenergic receptor kinase (βARK; Arden et al. 1995) or calcium/calmodulin-dependent protein kinase II (Koch et al. 1997). This occurs on a minute time scale. Desensitization is usually followed by sequestration and internalization of the receptor into endosomal vesicles. This is still a minute to hour-long procedure. Proteins in the endosomal vesicles can be recycled to the cell surface or degraded in lysosomes. On a longer time scale, down-regulation of the receptor can occur, meaning reduction of the total (surface and intracellular) receptor number. This certainly involves much more complicated regulatory steps in the gene expression, translation and/or degradation of the certain protein. The above-mentioned steps might give rise to the pharmacological phenomenon of tolerance, meaning that the same dose of the drug becoming ineffective in evoking a given response on repeated administration, or conversely, an even larger dose of drug is necessary to achieve the same magnitude of effect. The term dependence refers more to physiological (or somatic) and psychological aspects of addiction, the former characterized by withdrawal symptoms on the cessation of drug administration, and the latter by drug-seeking behavior. The different anatomical correlates and molecular mechanisms responsible for the opiate dependence have been reviewed by Nestler (1992, 1994, 1996).

As mentioned above in the discussion of the possibility of receptor oligomerization, opioid receptors are regulated in a ligand-specific manner (Burford et al. 1998; Keith et al. 1998; Allouche et al. 1999; Li et al. 1999). For example, the agonists DAMGO and endomorphin-1, but not morphine, caused μ receptor internalization, even though they were similar in activating individual G-proteins. Since endocytosis is associated with functional desensitization of receptor-mediated signal transduction, the differential effects of opiate drugs on this regulatory mechanism may be of great physiological importance. Whistler et al. (1999) suggest that the ability of a drug to induce opioid receptor endocytosis is an independent functional property of agonists, and they introduce the RAVE factor (relative activity versus endocytosis) as a measure of this. If the peptide agonist DAMGO is defined as having a RAVE value of 1, morphine has an approximately 4 times greater RAVE value, showing that its relative ability to signal is much higher than its relative ability to induce receptor endocytosis. They also hypothesized that, in contrast with the prevailing hypothesis, the failure of morphine-activated receptors to uncouple from G-protein and endocytose appropriately might be responsible for the high tolerance induced by this alkaloid.

However, not only receptors take part in the manifestation of tolerance and dependence, but also other elements of the signal transduction pathway. Although most emphasis has been placed on analysis of the internalization and redistribution of GPCRs, it has also been recognized that sustained agonist treatment of cells can result in alterations in both the cellular distribution and levels of G-proteins activated by the relevant GPCR (Nestler et al. 1989; Terwilliger et al. 1991; Van Vliet et al. 1993; Selley et al. 1997). Exposure of cells to agonists of receptors linked to G-proteins can the result in up- or downregulation of cellular levels or redistribution of G-proteins from membranes to the cytosol. Agonist-induced reductions in G-protein levels have been observed for members of each of the G_s, G_i and G_q families of G-proteins, are likely to be dependent upon the level of receptor expression or the brain area investigated, and are generally restricted to the G-protein(s) with which the receptor interacts. The mechanisms responsible vary with cell type and include both second messenger-dependent and -independent enhanced protein degradation. An agonist-induced reduction in cellular G-protein levels can provide one mechanism for the development of sustained heterologous desensitization (for a review, see Milligan 1993). Selective upregulation of certain G-proteins after chronic opioid treatment has also been reported (Escriba et al. 1994; Manji et al. 1997). The distinct pattern of changes in G-protein subtypes detected after morphine administration might represent different stages of the cellular adaptation to the continuous presence of the drug and might reflect different roles of the G-protein subtypes in this process. These data fit into the scheme of drug regulation of neuronal gene expression suggested by Nestler (1992, 1994), where one main group of genes targeted by the drug effect is that encoding G-proteins. The altered gene expressions of several components of the cell signaling system, such as adenylyl cyclase (Avidor-Reis et al. 1996; Rivera and Gintzler 1998), protein kinase-C (PKC; Ventayol et al. 1997), G-protein coupled receptor kinase (Ozaita et al. 1998) and protein phosphatases (Bernstein and Welch 1998), contribute to the neuronal plasticity.

Conclusions

Data obtained by the use of molecular biological tools prove that heterotrimeric G-proteins are not merely simple on/off switches of effector functions, but also active integrators of different intracellular processes. They provide several stages for the fine-tuned regulation of the cellular functions induced by different extracellular stimuli. The number of known members of the RGS family will definitely increase rapidly in the future, probably including proteins with effects different from the activation of GTP hydrolysis. Having several protein-protein interaction domains, G-proteins can regulate the compositions of molecular complexes formed after ligand-receptor activation, recruiting components known previously to belong to separated signaling pathways.

As we begin to understand the detailed molecular mechanisms involved in the signaling of opioid receptors, the complexity is becoming increasingly evident. The heterogeneity of the receptors (μ, δ, κ and several subtypes in these classes) and of the signal transducer G-proteins interacting

with them is further complicated by the variety of downstream elements of different signaling cascades. This wide array of interactions and regulatory effects might provide the basis of the unique properties of the opioid ligands in inducing heavy addiction in drug users. The clue to the prevention of the manifestation of tolerance in the clinical use of opioids or the successful therapy of opioid-dependent patients lies in identification of the particular signaling complexes implicated in the post-receptor events.

References

Ahnert-Hilger G, Schafer T, Spicher K, Grund C, Schultz G, Wiedenmann B (1994) Detection of G-protein heterotrimers on large dense core and small synaptic vesicles of neuroendocrine and neuronal cells. Eur J Cell Biol 65(1):26-38.

Allouche S, Polastron J, Hasbi A, Homburger V, Jauzac P (1999) Differential G-protein activation by alkaloid and peptide opioid agonists in the human neuroblastoma cell line SK-N-BE. Biochem J 342(Pt 1):71-78.

Arden JR, Segredo V, Wang Z, Lameh JH, Sadee W (1995) Phosphorylation and agonist-specific intracellular trafficking of an epitope-tagged mu-opioid receptor expressed in HEK 293 cells. J Neurochem 65:1636-1645.

Avidor-Reis T, Nevo I, Levy R, Pfeuffer T, Vogel Z (1996) Chronic opioid treatment induces adenylyl cyclase V superactivation. Involvement of G-betagamma. J Biol Chem 271:21309-21315.

Bem WT, Yeung SJ, Belcheva M, Barg J, Coscia CJ (1991) Age-dependent changes in the subcellular distribution of rat brain μ-opioid receptors and GTP binding regulatory proteins. J. Neurochem 57:1470-1477.

Benyhe S, Farkas T, Wollemann M (1989) Effects of sodium on [^{3}H]ethylketocyclazocine binding to opioid receptors in frog brain membranes. Neurochem Res 14:205-210.

Benyhe S, Szűcs M, Varga E, Simon J, Borsodi A, Wollemann M (1989) Cation and guanine nucleotide effects on ligand binding properties of mu and delta opioid receptors in rat brain. Acta Biochim Biophys Hung 24:69-81.

Benyhe S, Varga E, Hepp J, Magyar A, Borsodi A, Wollemann M (1990) Characterization of kappa$_1$ and kappa$_2$ opioid binding sites in frog (*Rana esculenta*) brain membrane preparation. Neurochem Res 15:899-904.

Bernstein MA, Welch SP (1998) Inhibition of protein phosphatases alters the expression of morphine tolerance in mice. Eur J Pharmacol 341:173-177.

Birnbaumer L (1992) Receptor-to-effector signaling through G proteins: Roles for βγ dimers as well as α subunits. Cell 71:1069-1072.

Blume AJ, Lichtshtein D, Boone G (1979) Coupling of opiate receptors to adenylate cyclase: requirement for Na$^+$ and GTP. Proc Natl Acad Sci USA 76:5626-5630.

Bomsel M, Mostov K (1992) Role of heterotrimeric G proteins in membrane traffic. Mol Biol Cell 3:317-328.

Borea PA, Bertelli GM, Gilli G (1988) Temperature dependence of the binding of μ, δ and κ agonists to the opiate receptors in guinea-pig brain. Eur J Pharmacol 146:247-252.

Bourne HR, Sanders DA, McCormic F (1990) The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125-132.

Bourne HR, Sanders DA, McCormic F (1991) The GTPase superfamily: a conserved structure and molecular mechanism. Nature 349:117-127.

Bozó B, Farkas J, Tóth G, Wollemann M, Szűcs M, Benyhe S. (2000) Receptor binding and G-protein activation by new Met5-enkephalin-Arg6-Phe7 derived peptides. Life Sci 66:1241-1251.

Brett J, Gerlach H, Nawroth P, Steinberg S, Godman G, Stern D (1989) Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. J Exp Med 169:1977-1991.

Burchett SA (2000) Regulators of G protein signaling: a bestiary of modular protein binding domains. J Neurochem 75:1335-1351.

Burford NT, Tolbert LM, Sadée W (1998) Specific G protein activation and μ-opioid receptor internalization caused by morphine, DAMGO and endomorphin I. Eur J Pharmacol 342:123-126.

Carrasco MA, Sierralta J, Mazancourt P (1994) Characterization and subcellular distribution of G-proteins in highly purified skeletal muscle fractions from rabbit and frog. Arch Biochem Biophys 310:76-81.

Chen Y, Mestek A, Liu J, Hurley JA, Yu L (1993) Molecular cloning and functional expression of rat μ-opioid receptor from rat brain. Mol Pharmacol 44:8-12.

Childers SR (1991) Opioid receptor-coupled second messenger systems. Life Sci 48:1991-2003.

Collins S, Caron MG, Lefkowitz RJ (1992) From ligand binding to gene expression: new insights into the regulation of G-protein-coupled receptors. Trends Biochem Sci 17:37-39.

Colombo MI, Mayorga LS, Nishimoto I, Ross EM, Stahl PD (1994) Gs regulation of endosome fusion suggests a role for signal transduction pathways in endocytosis. J Biol Chem 269(21):14919-14923.

Cvejic S, Devi LA (1997) Dimerization of the delta opioid receptor: implication for a role in receptor internalization. J Biol Chem 272:26959-26964.

Escriba PV, Sastre M, Garcia-Sevilla JA (1994) Increased density of guanine nucleotide-binding proteins in the postmortem brains of heroin addicts. Arch General Psychiatry 51:494-501.

Evans CJ, Keith DE, Morrison H, Magendzo K, Edwards RH (1992) Cloning of a delta opioid receptor by functional expression. Science 258:1952-1955.

Fábián G, Szabó C.A, Bozó B, Greenwood J, Adamson P, Deli MA, Joó F, Krizbai IA, Szűcs M (1998) Expression of G-protein subtypes in cultured cerebral endothelial cells. Neurochem Int 33:179-185.

Fábián G, Benyhe S, Farkas J, Szűcs M (1996) Thermodynamic parameters of opioid binding in the presence and absence of G-protein coupling. J Rec and Signal Transd Res 16:151-168.

Gaveriaux-Ruff C, Peluso J, Befort K, Simonin F, Zilliox C, Kieffer BL (1997) Detection of opioid receptor mRNA by RT-PCR reveals alternative splicing for the delta- and kappa-opioid receptors. Brain Res Mol Brain Res 48:298-304.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. Ann Rev Biochem 56:615-649.

Goodman RH (1990) Regulation of neuropeptide gene expression. Annu Rev Neurosci 13:111-117.

Green JM, Zhelesnyak A, Chung J, Lindberg FP, Sarfati M, Frazier WA, Brown EJ. (1999) Role of cholesterol in formation and function of a signaling complex involving alphavbeta3, integrin-associated protein (CD47), and heterotrimeric G proteins. J Cell Biol 146:673-682.

Gudermann T, Schöneberg T, Schultz G (1997) Functional and structural complexity of signal transduction via G-protein-coupled receptors. Annu Rev Neurosci 20:399-427.

Hall A (1990) The cellular functions of small GTP-binding proteins. Science 249:635-639.

Helms JB (1995) Role of heterotrimeric GTP binding proteins in vesicular protein transport: indications for both classical and alternative G protein cycles. FEBS Lett 369:84-88.

Hepler JR, Gilman AG (1992) G-proteins. Trends Biochem Sci 17:383-387.

Hintzemann R, Murphy M, Curell J (1985) Opiate receptor thermodynamics: agonist and antagonist binding. Eur J Pharm 108:171-177.

Holz GG, Turner TJ (1998) Pertussis toxin-sensitive GTP-binding proteins characterized in synaptosomal fractions of embryonic avian cerebral cortex. Comp Biochem Physiol 119B:201-211.

Hoyer J, Popp R, Meyer J, Galla HJ, Gogelein H (1991) Angiotensin II, vasopressin and GTP[gamma-S] inhibit inward-rectifying K+ channels in porcine cerebral capillary endothelial cells. J Membr Biol 123:55-62.

Johansson L, Persson H, Rosengren E (1992) The role of Mg^{2+} on the formation of the ternary complex between agonist, β-adrenoceptor, and

G_s-protein and an interpretation of high and low affinity binding of β-adrenoceptor agonists. Pharmacol Toxicol 70:192-197.

Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proc Natl Acad Sci USA 98:343-348

Jordan BA, Cvejic S, Devi LA (2000) Opioids and their complicated receptor complexes. Neuropsychopharmacology 23:S5-S18

Kaziro Y, Itoh H, Kozasa T, Nakafuku M, Satoh T (1991) Structure and functions of signal-transducing GTP-binding proteins. Annu Rev Biochem 60:349-400.

Keith DE, Anton B, Murray SR, Zaki PA, Chiu PC, Lissin DV, Monteillett-Agius G, Stewart PJ, Evans C,, von Zastrow M (1998) μ-opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism *in vivo* and *in vitro*. Mol Pharmacol 53:377-384.

Kieffer BL, Befort K, Gaveriaux-Ruff C, Hirth CG (1992) The delta-opioid receptor: Isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 89:12048-12052.

Koch T, Schulz S, Schroder H, Wolf R, Raulf E, Hollt V (1998) Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization. J Biol Chem 273:13652-13657.

Koch T, Kroslak T, Mayer P, Raulf E, Hollt V (1997) Site mutation in the rat mu-opioid receptor demonstrates the involvement of calcium/calmodulin-dependent protein kinase II in agonist-mediated desensitization. J Neurochem 69:1767-1770.

Ktistakis NT, Linder ME, Roth MG (1992) Action of brefeldin A blocked by activation of a pertussis-toxin-sensitive G protein. Nature 356:344-346.

Laduron PM (1994) From receptor internalization to nuclear translocation. New targets for long-term pharmacology. Biochem Pharmacol 47:3-13.

Laduron PM (1992) Genomic pharmacology: more intracellular sites for drug action. Biochem Pharmacol 44:1233-1242.

Lanoix J, Roy L, Paiement J (1989) Detection of GTP-binding proteins in purified derivatives of rough endoplasmic reticulum. Biochem J 262:497-503.

Laugwitz K-L, Offermanns S, Spicher K, Schultz G (1993) Mu- and delta-opioid receptors differentially couple to G-protein subtypes in membranes of human neuroblastoma SH-SY5Y cells. Neuron 10:233-242.

Li J-G, Luo J-Y, Krupnick JG, Benovic JL, Liu-Chen L-Y (1999) U50,488H-induced internalization of the human κ opioid receptor involves a β-arrestin- and dynamin-dependent mechanism. J Biol Chem 274:12087-12094.

Linder ME, Pang IH, Duronio RJ, Gordon JI, Sternweis PC, Gilman AG (1991) Lipid modifications of G protein subunits: Myristoylation of $G_{o\alpha}$ increases its affinity for βγ. J Biol Chem 271:8772-8778.

Manji HK, Chen G, Potter W, Kosten TR (1997) Guanine nucleotide binding proteins in opioid-dependent patiens. Biol Psychiatry 41:130-134.

Martin WR, Eades CC, Thompson JA, Gilbert PE, Huppler RE (1976) The effect of morphine and nalorphine-like drugs in the nondependent and morphine dependent chronic spinal dog. J Pharmacol Exp Ther 197:517-532.

McKenzie FR, Milligan G (1990) Delta-opioid-receptor-mediated inhibition of adenylate cyclase is transduced specifically by the guanine-nucleotide-binding protein Gi2. Biochem J 267:391-398.

Milligan G (1993) Agonist regulation of G protein levels and distribution: mechanisms and functional implications. Trends Pharmacol Sci 14:413-418.

Milligan G (1996) The stoichiometry of expression of protein components of the stimulatory adenylyl cyclase cascade and the regulation of information transfer. Cell Signal 8:87-95.

Montminy MR, Gonzalez GA, Yamamoto KK (1990) Regulation of cAMP-inducible genes by CREB. Trends Neurosci 13:184-188.

Morishita R, Nakayama H, Isobe T, Matsuda T, Hashimoto Y, Okano T, Fukada Y, Mizuno K, Ohno S, Kozawa O, Kato K, Asano T (1995) Primary structure of a γ subunit of G protein, γ_{12}, and its phosphorylation by protein kinase C. J Biol Chem 270:29469-29475.

Mumby SM, Heukeroth RO, Gordon JI, Gilman AG (1990) G protein a subunit expression, myristoylation and membrane association in COS cells. Proc Natl Acad Sci USA 87:728-732.

Nestler EJ, Erdos JJ, Terwilliger R, Duman RS, Tallman JF (1989) Regulation of G proteins by chronic morphine in the rat locus coeruleus. Brain Res 476:230-239.

Nestler EJ (1996) Under siege: The brain on opiates. Neuron 16:897-900.

Nestler EJ (1994) Molecular neurobiology of drug addiction. Neuropsychopharmacol 11:77-87.

Nestler EJ (1992) Molecular mechanisms of drug addiction. J Neurosci 12:2439-2450.

Nicolas P, Hammonds Jr, RG, Gomez S, Li CH (1982) β-Endorphin: Thermodynamics of the binding reaction with rat brain membranes. Arch Biochem Biophys 217:80-86.

Offermanns S, Schultz G, Rosenthal W (1991) Evidence for opioid receptor-mediated activation of the G-proteins, G_o and G_{i2}, in membranes of neuroblastoma x glioma (NG108-15) hybrid cells. J Biol Chem 266:3365-3368.

Ozaita A, Escriba PV, Ventayol P, Murga C, Mayor F Jr, Garcia-Sevilla JA (1998) Regulation of G protein-coupled receptor kinase 2 in brains of opiate-treated rats and human opiate addicts. J Neurochem 70:1249-1257.

Pierce KL, Luttrell LM, Lefkowitz RJ (2001) New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades. Oncogene 20:1532-1539.

Pimplikar SW, Simons K (1993) Regulation of apical transport in epithelial cells by a Gs class of heterotrimeric G protein. Nature 362:456-458.

Quock RM, Burkey TH, Varga E, Hosohata Y, Hosohata K, Cowell SM, Slate CA, Ehlert FJ, Roeske WR, Yamamura HI (1999) The delta-opioid receptor: molecular pharmacology, signal transduction, and the determination of drug efficacy. Pharmacol Rev 51:503-532.

Raub TJ (1996) Signal transduction and glial cell modulation of cultured brain microvessel endothelial cell tight junctions. Am J Physiol 271:C495-503.

Ray K, Kunsch C, Bonner LM, Robishaw JD (1995) Isolation of cDNA clones encoding eight different human G protein γ subunits, including three novel forms designated the γ_4, γ_{10}, and γ_{11} subunits. J Biol Chem 270:21765-21771.

Raynor K, Kong H, Law S, Heerding J, Tallent M, Livingston F, Hines J, Reisine T (1996) Molecular biology of opioid receptors. NIDA Res Monogr 161:83-103.

Rivera M, Gintzler AR (1998) Differential effect of chronic morphine on mRNA encoding adenylyl cyclase isoforms: relevance to physiological sequela of tolerance/dependence. Brain Res Mol Brain Res 54:165-169.

Robinson MS, Kreis TE (1992) Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. Cell 69:129-138.

Rottmann M, Benyhe S, Szűcs M (1994) Guanine nucleotide and cation modulation of [^{3}H]ethylketocyclazocine binding in frog brain membranes. Regul Peptides S23-S25.

Seasholtz TM, Majumdar M, Brown HJ (1999) Rho as a mediator of G protein-coupled receptor signaling. Mol Pharmacol 55:949-956.

Selbie LA, Hill SJ (1998) G-protein-coupled-receptor cross-talk: the fine tuning of multiple receptor-signalling pathways. Trends Pharmacol Sci 19:87-98.

Selley DE, Nestler EJ, Breivogel CS, Childers SR (1997) Opioid receptor coupled G-proteins in rat locus coeruleus membranes: decrease in activity after chronic morphine treatment. Brain Res 746:10-18.

Simon J, Szűcs M, Benyhe S, Borsodi A, Zeman P, Wollemann M (1984) Solubilization and characterization of opioid binding sites from frog (*Rana esculenta*) brain. J Neurochem 43:957-963.

Snow BE, Krumins AM, Brothers GM, Lee SF, Wall MA, Chung S, Mangion J, Arya S, Gilman AG, Siderovski DP (1998) A G protein

gamma subunit-like domain shared between RGS11 and other RGS proteins specifies binding to Gbeta5 subunits. Proc Natl Acad Sci USA 95:13307-13312

Standifer, KM, Pasternak, GW (1997) G proteins and opioid receptor-mediated signalling. Cell Signal 9:237-248.

Schulz S, Schreff M, Koch T, Zimprich A, Gramsch C, Elde R, Hollt V (1988) Immunolocalization of two mu-opioid receptor isoforms (MOR1 and MOR1B) in the rat central nervous system. Neurosci 82: 613-622.

Surrat CK, Johnson PS, Moriwaki A, Seidleck BK, Blaschak CJ, Wang JB, Uhl GR (1994) μ opiate receptor. Charged transmembrane domain amino acids are critical for agonist recognition and intrinsic activity, J Biol Chem 269:20548-20553.

Szűcs M, Coscia CJ (1992) Differential coupling of opioid binding sites to guanosine triphosphate binding regulatory proteins in subcellular fractions of rat brain. J Neurosci Res 31:565-572.

Szűcs M, Spain JW, Oetting GM, Moudy AM, Coscia CJ (1987) Guanine nucleotide and cation regulation of μ, δ and κ opioid receptor binding: Evidence for differential postnatal development in rat brain. J Neurochem 48:1165-1170.

Terwilliger RZ, Beitner-Johnson D, Sevarino KA, Stanley NC, Nestler EJ (1991) A general role for the adaptations in G proteins and cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function. Brain Res 548:100-110.

Toki C, Oda K, Ikehara Y (1989) Demonstration of GTP-binding proteins in rat liver Golgi fraction. Biochem Biophys Res Comm 164:333-338.

Van Vliet BJ, Van Rijswijk ALCT, Wardeh G, Mulder AH, Schoffelmeer ANM (1993) Adaptive changes in the number of Gs- and Gi- proteins underlie adenylyl cyclase sensitization in morphine-treated rat striatal neurons. Eur J Pharmacol 245:23-29.

Ventayol P, Busquets X, Garcia-Sevilla JA (1997) Modulation of immunoreactive protein kinase C-alpha and beta isoforms and G proteins by acute and chronic treatments with morphine and other opiate drugs in rat brain. Naunyn Schmiedeberg's Arch Pharmacol 355:491-500.

Vogel SS, Chin GJ, Schwartz JH, Reese TS (1991) Pertussis toxin-sensitive G proteins are transported toward synaptic terminals by fast axonal transport. Proc Natl Acad Sci USA 88:1775-1778.

Watson AJ, Katz A, Simon MI (1994) A fifth member of the mammalian G protein β-subunit family. J Biol Chem 269:22150-22156.

Watson N, Linder ME, Druey KM, Kehrl JH, Blumer KJ (1996) RGS family members: GTPase activating proteins for heterotrimeric G-protein α-subunits. Nature 383:172-175.

Wedegaertner PB, Bourne HR (1994) Activation and depalmitoylation of Gsα. Cell 77:1063-1070.

Weiland T, Schulze R, Jakobs KH (1997) Heterotrimeric guanine nucleotide binding proteins: structure and function. In Wirtz KWA, ed., Molecular mechanisms of signalling and membrane transport. NATO ASI Series, Vol. H 101.

Whistler JL, Chuang HH, Chu P, Jan LY, von Zastrow M (1999) Functional dissociation of mu opioid receptor signaling and endocytosis: implications for the biology of opiate tolerance and addiction. Neuron 23:737-746.

Wollemann M, Benyhe S, Simon J (1993) The kappa-opioid receptor: evidence for different subtypes. Life Sci 52:599-611.

Yasuda K, Raynor K, Kong H, Breder CD, Takeda J, Reisine T, Bell GI (1993) Cloning and functional comparison of κ and δ opioid receptors from mouse brain. Proc Natl Acad Sci USA 90:6736-6740.

Zarbin MA, Palacios JM, Wamsley JK, Kuhar MJ (1983) Axonal transport of beta-adrenergic receptors. Antero- and retrogradely transported receptors differ in agonist affinity and nucleotide sensitivity. Mol Pharmacol 24:341-348.

Zarbin MA, Wamsley JK, Kuhar MJ (1990) Anterograde transport of opioid receptors in rat vagus nerves and dorsal roots of spinal nerves: pharmacology and sensitivity to sodium and guanine nucleotides. Exp Brain Res 81:267-278.

Zastrov MV, Keith DE, Evans CJ (1993) Agonist-induced state of the opioid receptor that discriminates between opioid peptides and opiate alkaloids. Mol Pharmacol 44:166-172.

Zazopoulos E, De Cesare D, Foulkes NS, Mazzucchelli C, Lamas M, Tamai K, Lalli E, Fimia G, Whitmore D, Heitz E, Sassone-Corsi P (1997) Coupling signal transduction to transcription: the nuclear response to cAMP. In Wirtz KWA, ed., Molecular mechanisms of signalling and membrane transport. NATO ASI Series, Vol. H 101.

Zeman P, Tóth G, Kvetnansky R (1997) Thermodynamic analysis of rat brain opioid mu-receptor-ligand interaction. Gen Physiol Biophys 6:237-248.

Volume 45(1-4):23-42, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

REVIEW ARTICLE

Normal and abnormal development of visual functions in children

Petra Kozma[1,2]*, Ilona Kovács[3], György Benedek[1]

[1]Department of Physiology, [3]Department of Ophthalmology, University of Szeged, Szeged, Hungary, [2]Laboratory of Vision Research, Rutgers University, NJ, USA

ABSTRACT The human visual system goes through substantial changes during the first few months of postnatal life. The development of visual functions and structures occurs at different times and different rates. It has been a generally held belief that the development of visual functions and their critical period come to an end early in life. Most of the developmental data confirm this theory, although the findings sometimes are contradictory. Thus, our knowledge concerning visual development does not seem to be complete. The determination of exact timing of the different visual functions is relevant in children since a proved extended maturational timeframe can promote the trial of enhancement of visual abilities at a later age, up to puberty or beyond. There have already been suggestions for an extended developmental time span for some of the visual functions. Here we review the most relevant data with reference to the normal development of the eye, visual functions and visual pathways found in the literature and provide further evidence for the maturation and plasticity of visual functions after the age of 5 years.

Acta Biol Szeged 45(1-4):23-42 (2001)

KEY WORDS

visual development
critical period
contour-integration
amblyopia
plasticity

The need for understanding visual development has received increasing attention in the last four decades (Fiorentini 1984) since it is a system where work at behavioral, anatomical and physiological levels can be correlated to lead to study the basic mechanisms involved (Daw 1994).

Until the early 1960s little was known about the anatomical and functional properties of the visual system of the newborn and of its subsequent development during infancy. A new impulse to the investigation of visual development was given by the classical, pioneering single cell studies of Hubel and Wiesel on cats who received the Nobel Prize in Medicine in appreciation of their work in 1981 (Hubel and Wiesel 1963a, b). Hubel and Wiesel deprived one or both eyes of visual impulses from birth in cats. They found more significant structural and morphological changes of the visual pathways during closure of one eye than during the closure of both eyes (Wiesel and Hubel 1965). On the other hand, closure had no effect on the eyes and on the cells in the visual cortex of adult cats. Accordingly, Hubel and Wiesel were the first who described and termed the "critical period" of vision. The critical period is a definite period of time, early in life during which the visual system is plastic and is susceptible to environmental influence as well as to abnormal visual experience (Hubel and Wiesel 1970; Barlow 1975; Wiesel 1982; Daw 1994). If stimulus deprivation occurs during this period, visual development will be impaired. Since the work of Hubel and Wiesel there has been an explosion of morphological, electrophysiological and behavioral experiments that studied how postnatal visual development occurs and whether and how it could be affected by early visual deprivation or by manipulations of the visual environment (Fiorentini 1984). Thus, the visual system has become the model for the understanding of plasticity (Daw 1994).

The study of vision in human infants has progressed considerably in parallel with the animal studies (Fiorentini 1984). It was shown that critical periods also exist in the visual development of humans. The clearest demonstration of this similarity, came out in studies on kittens and young monkeys with experimentally induced strabismus based on the pioneering work of Wiesel and Hubel (1963a, b).

The human visual system is immature at birth both anatomically and functionally and goes through substantial changes especially during the first few months of postnatal life (Atkinson 1984; Fiorentini 1984; Garey 1984). The development of the visual cortex occurs in a hierarchical order. The critical period seems to vary in onset and duration between different brain regions and even between layers of an individual cortical area. Lower levels of the visual system and deeper layers of the cortex mature earlier compared to the higher and more superficial ones (Conel 1939-1967; Harwerth et al. 1986; Daw 1994). Different functions may emerge at different times and develop at different rates (Levi

Accepted December 5, 2000
*Corresponding author. E-mail: pkozma@matavnet.hu

and Carkeet 1993). The general belief that the critical period terminates by the end of the second year of life has been kept for long (Wright 1995). Most of the developmental data (see Normal visual development) until recently have confirmed this theory, although they are somewhat controversial. There are suggestions for an extended maturational time frame of some of the visual functions.

Therefore, the exact timing of the maturation of human visual functions and thus the length of plasticity and the so called critical period have remained to be a question. The accurate settling of the critical period would give the opportunity to more children for functional recovery, since impaired visual functions and developmental disorders *e.g.* amblyopia can be restored with a good chance before their critical period is over (Daw 1995). The lack of evidence in this field can be due to poor compliance of young children and the absence of standard and reliable evaluaton as well as research methods for children.

In the view of the above mentioned notions the aim of our studies was two fold. On the one hand, to determine the length of the critical period in normal visual development by examining a less known visual function, namely, the spatial integration ability of children in different age groups through adolescence. Thus, to support or defeat the current belief that "the maturation of the perceptual functions completes by the age of two and cognitive development can be expected afterwards". On the other hand, to study what type of changes can occur in children during the assumed critical period of a visual disorder called "amblyopia" when the treatment is considered to be late. To determine if it is worth performing late surgery at all and if we can expect functional changes besides cosmetical benefits.

Normal visual development

Substantial changes and rapid visual development occur during the first 6 months of postnatal life. Infants probably can discriminate between colors (Bornstein et al. 1976; von Noorden 1985; Burr et al. 1996), have preference for moving stimuli (Nelson and Horowitz 1987) and can process complex motion information (Kellman and Spelke 1983) by 3-4 months of age. Optokinetic nystagmus (OKN; Atkinson 1979, 1984; Nagele and Held 1982; Lewis et al. 1989; Eustis 1995), saccadic eye movements (McGinnis 1930; Barten et al. 1971; Atkinson 1984) and fixation are present from birth (Dayton et al. 1964b; Isenberg 1989; Eustis 1995) but become mature only by 6 months of age. Depth perception, discrimination (Aslin 1977; Braddick et al. 1980; Teller 1982; Atkinson 1984), smooth pursuit (Dayton and Jones 1964a; Atkinson 1984; Isenberg 1989; Johnson 1990) and eye alignment (Eustis 1995) develop to full maturity also around the age of 6 months. Although visual acuity, stereopsis and contrast sensitivity emerge and improve dramatically within the first 6 months after birth (Atkinson et al. 1981; Atkinson 1984), they reach adult levels sometime between 1 and 5 years of age (McGinnis 1930; Dobson and Teller 1978; Gwiazda et al. 1980; Birch et al. 1983), 3 and 9 years of age (Romano et al. 1975; Braddick et al. 1980; Fox et al. 1980, 1986, Held et al. 1980; Birch et al. 1982) and by 2 to 5 years (Atkinson et al. 1981; Bradley and Freeman 1982) of age individually. Binocular vision and fusion also emerge around 1.5-4 months (Braddick et al. 1980; Petrig et al. 1981; Braddick and Atkinson 1983; Leguire et al. 1991) and mature between 1 to 7 years of age (Banks and Aslin 1975; Hohmann and Creutzfeld 1975). Based on the findings of visual evoked potential studies there are some suggestions that adult like acuities can already be observed in 4 to 7 months old infants (Marg et al. 1976; Eustis 1995). The visual field is supposed to reach adult values between 6 months (Mayer et al. 1988; Lewis and Maurer 1992) and 5 years (Lakowski and Aspinall 1969; Matsuo et al. 1974) depending on the technique and stimulus applied (Lewis and Maurer 1992; Sireteanu 1996a). Wilson et al. (1991), however, reported a much slower maturation that lasts through 10 years of age. A slower development of contrast sensitivity was found by Beazley et al. (1980) also up to early adolescence.

The ocular media are clear from birth (Atkinson 1984). The intraocular and orbital structures seem to be well developed at birth, however dramatic morphological, anatomical and physiological changes occur in them during infancy and continue in the first few years of life (Ozanics and Jakobiec 1985). The most significant changes happen in the course of the first 6 to 12 months, but the development *e.g.* in the volume of the orbit can last up to 6-8 years of age while in the case of the eyeball it continues to mature until around 13 years of age (Blomdahl 1979; Swan and Wilkins 1984; Gordon and Donzis 1985).

At birth the human visual system is immature at the level of retina, lateral geniculate nucleus (LGN), and visual cortex, too (Garey 1984). The retina develops intensively during the first 6 months of life and comes to its full maturity around age 1-4 years (Abramov et al. 1982; Hendrickson and Yuodelis 1984; Yuodelis and Hendrickson 1986). The LGN reaches adult volume by the end of the first 6 months (de Courten and Garey 1982; Huttenlocher et al. 1982; Garey and de Courten 1983), but the morphological maturation and the development of different visual functions occur between 8 months to 2 years (Hickey 1981; Huttenlocher et al. 1982; Garey and de Courten 1983; Garey 1984).

The primary visual pathway becomes functional around the age of 2-3 months (Bronson 1974, 1982). Recent data by Sloper and Collins (1998), however, demonstrated that the central visual pathways continue to mature after the age of 5. The myelinisation of the optic nerve lasts until 2 years of age (Marg et al. 1976; Fox et al. 1980; Johnson 1990). Some of the extrastriatal visual areas and intracortical interneurons probably have a much longer myelinisation period (Yakovlev and Lecours 1967; Atkinson 1984).

After birth, within 6 months to first year of life the morphology and the volume of the visual cortex changes rapidly. Recent studies, however, suggested that there is a continuous increase in the neuronal number until 6 years after birth (Shankle et al. 1998a). This can imply a prolonged structural maturation of the human visual cortex. At about 4 months of age the primary visual cortex (V1) reaches adult volume, much earlier than the brain as a whole (Lemire et al. 1975; Garey and de Courten 1983; Garey 1984; Huttenlocher and de Courten 1987). Adult values of synaptic density are reached at the age of 4 years in the primary, while at the age of 11 years (Huttenlocher 1994; Huttenlocher and Dabholkar 1997) in higher cortical areas. More recent functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) data suggest that the human cortex as well as the visual cortex has a prolonged development that involves structural changes and maturation even in adulthood (Chugani et al. 1987; Giedd et al. 1996; Sowell et al. 1999). An extended maturational period of visual functions has also been suggested by some groups who reported that children may have difficulties in recognition of incomplete objects (Gollin 1960) as well as in visual integration and form identification when it is based on contrasts in texture (Atkinson and Braddick 1992; Sireteanu and Rieth 1993), motion (Hollants-Gilhuijs et al. 1998a) or color (Hollants-Gilhuijs et al. 1998b).

Spatial integration

Spatial integration is our ability to segment the visual image and to perceive contours and borders of objects by the integration of the local features such as orientation, depth, color etc., across the visual field (Field et al. 1993; Kovács and Julesz 1993; Kovács et al. 1996a; Kovács 1996b; Dakin and Hess 1998; Pennefather et al. 1999). The efficiency of the integrating mechanism can be estimated psychophysically in a contour detection task that employs orientational noise (Field et al. 1993; Kovács and Julesz 1993; Kovács 1996b; Dakin and Hess 1998).

The world around us at a certain moment is very complex; it consists of many objects and surfaces at various distances that differ in their quality, color, contrast and have different meanings and importance for the observer. It is a basic issue in the history of psychology how object perception and recognition occur. Perception is a process of drawing meaning from the stimulation that reaches our sensory receptors. Perception of a stimulus may be affected by relations that exist between the stimulus and its apparent context and background. Grouping and segregation are crucial in the early stages of perception. Segregation occurs according to Gestalt (1910) principles (Kohler 1967): objects are grouped together, because they are close (proximity), have similar features (similarity), they follow the same direction (good continuation), move in the same direction (common fate), have symmetry or form continuous, enclosed contours (closure). Our perception is dominated by contours, which are fundamental to perceiving an object's shape (Sireteanu and Fronius 1981; Schiffman 1996).

Contour integration is part of the segregation process. The Gestalt laws that are mostly influence contour integration are good continuation (Field et al. 1993; isolinear paths of Gabor patches are easier to detect) and closure (Kovács and Julesz 1993). It is easier to detect a contour when it follows these laws. Pettet et al. (1998) reported that in addition to closure and good continuation, the geometric properties, *e.g.* alignment, were also substantial in contour detection.

Anatomical data show that the neuronal connections that give rise to this integration process are probably the long-range interactions (Mitchinson and Crick 1982; Nelson and Frost 1985; Ts'o and Gilbert 1988; Gilbert 1998). Thus, how we see the world depends very much on the organization of the neuronal circuits in the visual cortex and the synapses between cells within the cortex. The pattern of connectivity is determined as much by past experience as by actual stimulation. Their functional architecture seems to be dynamic and context dependent. The neuronal connections can either be facilitatory or inhibitory. Facilitation is a local process that can produce global activity through long-range interactions along a path when local constraints are met. The strength of the interactions is directly proportional to retinal distance. Facilitatory long-range horizontal intrinsic connections run over long distances in the visual cortex and interconnect cells with similar stimulus preferences. The cells in the primary visual cortex are sensitive to bars and edges of specific orientation and respond to stimuli from a certain location of the retina. These orientation selective cells facilitate one another when simultaneously stimulated and cause an enhanced response. Gabor functions, named after a Hungarian scientist Gábor Dénes, roughly model the receptive field structure of simple cells in V1 (Jones and Palmer 1987). Therefore, they are appropriate stimuli for the examination of these small spatial filters and their interactions in V1. Gabor patches are the products of a sine wave multiplied by a smooth bell shaped (Gaussian) envelope (Bruce et al. 1996). Gabor patches are widely used stimuli in contour detection tasks because they also lack edge cues, their contrast, size, spatial frequency, orientation, phase and location in space can be manipulated. Performance on contour detection task is dependent on orientation (Field et al. 1993) as well as on the parameters of Gabor patches. The contour and its surroundings also appear to influence contour detection (Bruce et al. 1996).

Long-range interactions probably refine postnatally in an experience-dependent fashion (Callaway and Katz 1990). The development of the long-range interactions depends on the maturational state of the layers within the primary visual cortex and follows a hierarchical pattern of development.

Burkhalter et al. (1993a) found that long-range interactions within layer 2/3 of the primary visual cortex develop after connections within layers 4B, 5 and 6. In layers 2/3 they are still immature even at 2 years of age in humans (Callaway and Katz 1990; Burkhalter 1993a; Polat and Sagi 1994). In the primary visual cortex vertical or intracolumnar connections that process local features of the visual field develop before horizontal or intercolumnar connections that are necessary for the integration of these local features into an image (Burkhalter et al. 1993a). There is also some psychophysical indication that the development of these connections in humans lasts longer — until around school age (Sireteanu and Rieth 1993) — than the development of other primary functions in infancy (Atkinson and Braddick 1992), but the exact age has not been determined yet.

Thus, it is essential to study contour detection developmentally. Work with infants may provide insight into mechanisms of cortical development. To study the human developmental pattern of spatial integration between orientation selective cells of the primary visual cortex we used a card test version (Kovács et al. 1996a; Pennefather et al. 1999) of a contour detection task in children (5-14 years) with normal vision. These developmental results will be reported below in details (see Maturation of visual spatial integration in children).

Abnormal visual development: amblyopia

Visual experience during development is necessary for normal vision. If visual experience early in life is abnormal a disorder called amblyopia develops. Children are most susceptible to the effects of abnormal visual experience between 9 months and 2 years of age, and sensitivity declines between 2 and 8 years of age (Vaegan and Taylor 1979; Sanke 1988; Daw 1995). Amblyopia together with strabismus is the most common functional visual disorders in early childhood (Levi and Carkeet 1993). Amblyopia is generally defined as reduced visual acuity — for diagnostic purposes at least 2 Snellen lines difference between the eyes (Kushner 1988) — of usually one eye that occurs in the absence of ocular structural abnormalities and is due to abnormal visual experience early in life. Strabismus or squint is the misalignment of the visual axes. It has two horizontal types: eso- (inward deviation) and exotropia (outward deviation). Esotropia is not controlled by fusional mechanisms and can lead to amblyopia if not treated properly (Hecht et al. 1996). Esotropias belong to two main groups: congenital or infantile, and acquired esotropias. When an esotropia occurs within 6 months after birth it is referred to as infantile and when it occurs beyond 6 months of age it is referred to as acquired esotropia (von Noorden 1985). From the aspect of our study acquired esotropia developed together with amblyopia will be in focus.

Amblyopia together with strabismus has always been an interesting issue during the last centuries. Its continuous timelessness is due to its high prevalence in the general population (1.0 to 4.0%; von Noorden 1985) as well as to its detrimental, long term effect on personality (Asbury and Burke 1995) and occupation of the amblyopes (Reinecke 1978). Amblyopia accounts for more cases of vision impairment than all other causes (ocular diseases and trauma) combined (von Noorden 1985, 1996). In those with strabismus or anisometropia, 40-60% develops amblyopia. Children with esotropia show an approximately four times greater incidence of amblyopia than those with exotropia (Costenbader et al. 1948).

Amblyopia is originated from the Greek words: amblys (blunt, dull) and opsia (eye, cheek, face, seeing). The term amblyopia was already known in ancient times, but it had another meaning: decreased vision. Le Cat was the first in the 17th century who provided the first clinical description of human amblyopia. Its real history began at that time. According to von Graefe's definition in 1888, it is the condition when "The doctor sees nothing and the patient sees very little." (von Noorden 1996). Many different definitions have evolved since the end of the 19th century but none of them seems to be perfect. Reduced visual acuity has only classically been regarded as the defining feature of amblyopia, since acuity represents solely one limit of the spatial visual capacity. Actually, functional amblyopia is a developmental disorder of spatial vision that is potentially reversible by occlusion therapy during a developmental critical period and associated with the presence of strabismus, anisometropia, or form deprivation early in life (Ciuffreda et al. 1991; von Noorden 1996; Kushner 1998). When strabismus and amblyopia develop together a condition called strabismic amblyopia occurs. The notion that amblyopia is a developmental disorder was first expressed by Worth in 1903. Functional amblyopia should be distinguished from organic amblyopia, which is poor vision caused by structural abnormalities of the eye or brain and irreversible to treatment.

The neural basis of amblyopia has been less thoroughly explored. What vision scientists claim has not been changed since the times of Javal who recognized in 1896 that the seat of anomaly in amblyopia lies centrally and its effect is not equally distributed across the retina. Recently, Kiorpes and Movshon (1996a) have found that the neural basis of amblyopia begins but does not end in V1. The primary effects may involve extrastriate visual areas, too (Kiorpes et al. 1996b).

Treatment

Amblyopia is difficult to detect, because it develops early in life. Treatment for amblyopia and strabismus nevertheless should be instituted as soon as the diagnosis is made to achieve the best possible outcome since it is only reversible within the so-called critical period. A paper by Sireteanu et

al. (1984), however, suggests a very unique and interesting finding that patching the good eye can improve several visual functions even after the critical period, and thus age is not a limiting factor in the initiation of the treatment. It was also shown that strabismic amblyopia may be reversible in adults who have lost the use of their good eye (Simon and Calhoun 1998). This finding also confirms the notion that critical period for cure of amblyopia lasts longer than the critical period for its creation (Daw 1995). The general rule of thumb is that every child under 9 years of age should undergo a trial of amblyopia therapy (Mitchell and Timney 1984; Greenwald and Parks 1990; Wright 1995).

The first step in treatment is the correction of any significant refractive error that may be present in the amblyopic eye (Reinecke 1978; Kushner 1998) by accurately prescribed spectacles that compensates for the full cycloplegic findings. The second and more important aspect of amblyopia therapy is occlusion or patching of the better eye that increases the number of cortical cells responding to the amblyopic eye. Occlusion therapy is continued until the vision of both eyes becomes equal or until no improvement has been noticed after a 3-months period of treatment (Simon and Calhoun 1998). Penalization, Cambridge vision stimulator (CAM) and pleoptics are alternatives to occlusion but do not work better than patching (Greenwald and Parks 1990). Other nonsurgical treatments of amblyopia include orthoptics and neurotransmitter, *e.g.* levodopa replacement (Pettigrew 1982; Gottlob and Stangler-Zuschrott 1990; Leguire et al 1992; von Noorden 1996). Surgery should probably wait until amblyopia has been treated. Although, it was reported that no significant difference was found in the outcome of surgery when amblyopia was fully or only partially treated (Lam et al. 1993). The aim of strabismus surgery is not only cosmetic but also has functional benefits, such as development of binocularity, reestablishment of the fusion reflex and prevention of sensor and motor complications. Weakening procedure is called recession, strengthening procedure is called resection. It has been shown that overall results are favorably influenced by early alignment of the eyes. Good eye alignment can be achieved in later years, but normal sensory adaptation (achievement of binocular fusion and stereoscopic depth perception) becomes more difficult as the child grows older (Reinecke 1978). By age 8, the sensory status is generally so fixed that it cannot be effectively influenced by treatment (Asbury and Burke 1995).

Visual functions

Visual functions that develop slowly seem most susceptible to the effects of abnormal visual input. In amblyopia the most prominent deficit is in spatial vision. The spatial visual performance of the amblyopic eyes resembles the performance of both immature and peripheral visual systems (Kiorpes 1992a; Levi and Carkeet 1993; Kiorpes and Movshon 1996a; Kiorpes et al. 1996b). Spatial resolution measured by either Snellen (optotype) or grating acuity, of the strabismic amblyopes is reduced in the central field but normal in the periphery (Sireteanu and Fronius 1981). When tested through the amblyopic eye, amblyopes also have decreased contrast sensitivity at high spatial and low temporal frequencies in the central visual field (Wali et al. 1991) and decreased visual discrimination ability (Vernier acuity or positional acuity; Eggers 1993; Levi and Carkeet 1993; von Noorden 1996). The grating acuity deficit is relatively small compared to Vernier acuity (Hess et al. 1990; Daw 1995; Kiorpes and Movshon 1996a). Dark adaptation in all (Hess et al. 1990) and color vision (Levi and Carkeet 1993; von Noorden 1996) in most of the amblyopic eyes are normal. Strabismic amblyopes appear to have an additional loss of positional uncertainty often accompanied by aberrations of space perception (mislocalize targets), and spatial distortion.

Binocular interactions are also abnormal in amblyopia. When amblyopia occurs early in life it can even result in lack of binocularity. In the absence of binocularity, fusion and stereopsis cannot evolve. In infantile and acquired esotropia, the sensorial component (fusion and stereopsis) of binocularity is impaired, while the motor part is not affected (Mitchell and Timney 1984). Fusion is an acquired reflex (Aslin 1977; Starger and Birch 1986). The potential advantages of fusion include improved stereoacuity, improvement in the development of fine motor skills (Rogers et al. 1982) and stability of ocular alignment (Arthur et al. 1989; Morris et al. 1993). It is widely believed that the development of fusion is rare if ocular alignment does not occur or is not stable enough during the critical period of development for binocular vision (Leguire et al. 1995).

In humans, according to clinical experience, binocularity is not fully established at birth and it is not functional until the second to fourth months of life (Hohmann and Creutzfeld 1975; Braddick et al. 1980; Petrig et al. 1981). If strabismus occurs during maturation it can lead to poor binocular vision (Mitchell and Timney 1984), but it has very little effect on binocularity after 6 to 8 years of age (Banks and Aslin 1975). However, there are some suggestions that fusional mechanisms may be modified even after the age of normal visual maturation (7 to 9 years of age; Roelfsema et al. 1994). Though there is no agreement about the exact timing of maturation it seems that binocular vision has the longest critical period (Konig et al. 1993; Simmers and Gray 1999), and the critical period of development is prolonged when amblyopia is present (Ciuffreda 1986).

If surgical treatment is performed before the age of five most congenital and acquired esotropic patients experience some level of binocularity and development of fusion postoperatively (Lam et al. 1993). The later a squint is acquired the more likely it is that binocular vision will be restored after a successful operation (Epelbaum et al. 1993; Kushner 1994;

Wright 1996; Kraft 1998). This is due to a greater initial opportunity for fusion development. The amount of fusion development depends on the time of the onset of strabismus. Thus, the debate about the timing of corrective surgery in acquired esotropia is less vivid; there is only a few data available on it in the literature. Early surgery, however, may be beneficial and also improves the chance of surgical success in acquired esotropia (Lam et al. 1993). In acquired strabismus early surgery refers mostly to the duration of squint before surgery. Under the age of 2 years surgery should be carried out within 3 months and after the age of 2 within 6 months from the onset to be considered early. Delay of surgical intervention tends to produce and strengthen unfavorable sensory and motor complications. In cases of congenital esotropia, early corrective surgery appears to be indicated for the development of cortical binocularity, that is presumably a prerequisite for fusion and stereopsis. In acquired strabismus, however, the entire argument for early realignment of eyes is to prevent those unfavorable complications and to return the eyes as quickly as possible to a position where fusion and fusion reflex can be reestablished.

Here we report our results on the effect of late strabismic surgery on binocularity and on pattern reversal visual evoked potentials (VEPs) in children suffering from acquired esotropia (see Effect of late strabismus surgery in children with acquired esotropia).

Visual evoked potentials

Most of our knowledge of amblyopia has been gained through subjective psychophysiological techniques and objective electrophysiological methods carried out on human amblyopes (Cibis 1975). Electrophysiological methods such as VEPs can differentiate more easily between abnormalities in retinal and cortical functions than psychophysical methods do. VEP is a gross electrical signal generated at the occipital cortex in response to visual stimulation (Phelps 1976; Carr and Siegel 1982). The electrical activity is recorded by scalp electrodes, which are usually placed midoccipitally (O_z, 10-20 system) about 1-3 cm above the inion (Carr and Siegel 1982). VEPs are typically recorded in response to flash of light or pattern stimuli. VEP pattern stimuli usually generated on an oscilloscope or on a video monitor are either phase reversed (also called pattern reversal, contrast reversal, or counterphase modulation) or flashed on and off. Pattern reversal checkerboards are the most commonly used stimuli in clinical settings (Bodis-Wollner et al. 1986). These consist of light and dark checks, that reverse periodically from black to white and back at a selected alternation rate while maintaining a constant mean luminance on the retina (Carr and Siegel 1982; Fishman and Sokol 1990). The field size, retinal location and specific stimulus parameters, such as pattern size, contrast, and rate of presentation of pattern stimuli can be varied (Fishman and Sokol 1990). Within the central region, the fovea and the parafovea can be stimulated differentially by varying check size. Bodis-Wollner et al. (1986) pointed out that checks of 10-15 minutes stimulate the fovea optimally, while larger checks, such as 50 minutes, stimulate parafoveal regions. (Fishman and Sokol 1990). VEP recordings require proper, constant fixation and concentration from the subjects since in their absence the observed VEP changes can easily be misinterpreted as clinically significant.

Waveforms of the pattern reversal VEPs were found to change rapidly during the first several months after birth from a broad, single slow positive component with a latency of 190 to 250 ms in infants to a simple triphasic negative-positive-negative complex: N80-P100-N145 (N1-P1-N2; Fig. 1). The most rapid changes occur during the first few months of life but gradual changes can be seen throughout the formative years. The first positive component (P100) at 100 ms emerges at different time for different check sizes (140'-17.5') from 2 weeks to 10 weeks after birth (Zhang et al. 1993). During maturation the N1-P1 amplitude becomes higher and P1 latency becomes shorter for all checksizes (Uysal et al. 1993). It is generally assumed that the shortening of VEP latency with age is due in part to the maturation of the myelination of the optic fibers (Fiorentini and Trimarchi 1992). It is generally agreed that latencies of pattern reversal VEPs to small checks (< 20; by 9 years) reach mature levels at a slower rate than to larger checks (by 3-4 months) (Moskowitz and Sokol 1983; Harding et al. 1989; Zhang et al. 1993). The N1P1 and P1N2 amplitudes however were not affected between 2 months to 9 years of age and were significantly higher than those of adults by 2 to 3 factors (Zhang et al. 1993). The maturation rate of VEPs probably differs according to the technique and stimulation applied. By approximately 3 months most normal infants give evidence of a binocular VEP (Atkinson 1984), which is due to the fact that binocular input to cortical neurons is not found until about 13 weeks on average (Braddick and Atkinson 1983; Braddick et al. 1986).

The P100 component is the most frequently studied component of the VEP. It is of macular origin (Halliday et al. 1979; Zhang et al. 1993; Crognale et al. 1997) and its amplitude and latency are frequently measured parameters in clinical studies at various spatial frequencies (check sizes) (Bodis-Wollner et al. 1981; Sokol 1983; Fishman and Sokol 1990). The latency improves while the amplitude decreases with increasing check size (Roy et al. 1995). Thus, the P100 component can be best elicited by small size checks (Bodis-Wollner et al. 1986) and seems to be the best indicator of binocular interaction. Shawkat et al. (1998) showed that the P100 component especially of the reversal VEP is also the most useful tool to differentiate normals from amblyopes. Differences between amplitude rates of amblyopes and normals decrease with increasing checksize and a similar effect can be seen between the amblyop and fellow eyes of

the amblyopes. Contrary to the flash VEP, the pattern VEP has been shown to be a sensitive detector of amblyopia, particularly when small (< 20-minute) checks are used (Arden et al. 1974; Sokol 1980, 1983; Fishman and Sokol 1990). The VEP amplitude of the first major positive wave (P1) in the amblyopic eye is reduced compared to the normal eye (Arden et al. 1974; Bodis-Wollner et al. 1986; Henc-Petrinovic et al. 1993), but the latency values are normal or only slightly increased (Sokol and Dobson 1976; Arden et al. 1979; Sokol 1980, 1983; Fishman and Sokol 1990). Accordingly, latency is not as sensitive a marker of amblyopia as that of amplitude (Fishman and Sokol 1990).

Summing up, VEP is a valuable, noninvasive clinical tool for assessing visual function, *e.g.* binocularity as well as for diagnosing amblyopia and predicting its treatment success in infants and preverbal children (Taylor and McCulloch 1992; Henc-Petrinovic et al. 1993).

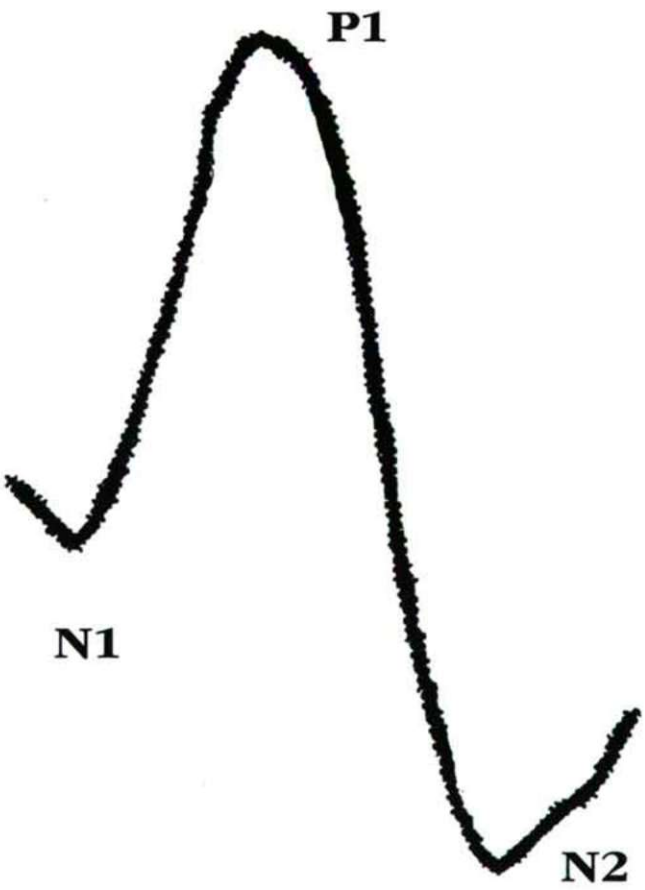


Figure 1. A VEP response. N1= negative peak 1; P1= positive peak 1 (P 100); N2= negative peak 2

Materials and Methods

Maturation of visual spatial integration in children

Three different experiments were conducted within this study. The development of spatial integration was tested in the "main study". The basis of the findings of the "main study" was tested in the "learning study" and in the "spatial range study".

Stimuli

Two different sets of cards were used: orientation- and color-defined (Fig. 2). The cards were generated on a Silicon Graphics Indy R4000 computer. Both the orientation-defined cards and the color-defined cards were printed on an Epson Stylus Color 800 printer. Gabor patches were used as stimuli on the orientation-defined cards. Carrier frequency of the Gabor patches was 5 c/deg at a 57 cm viewing distance, and their contrast was about 95%. Each card consisted of a closed chain of colinearly aligned Gabor patches (contour) and a background of randomly oriented and positioned Gabor patches (noise) (Fig. 2a, b).

Spacing between elements along the contour and spacing in the background were controlled independently. The algorithm allowed us to keep the smallest permitted separation between background elements while avoiding spurious spacing. At small signal-to-noise ratios, background elements were allowed to get into the spaces between contour elements, but orientation alignment was avoided. A new random shape and background were computed for each card. The length of the contours was constant, and the contours had a continuously positive curvature with no inflection points. Contour spacing was kept constant (7 l, where l = wavelength of Gabor patches) with increasing background density across the cards. The value of D (relative noise density), where D = noise spacing/contour spacing, defined the difficulty level of each card. D was varied across cards in 0.05 stepsize. Variations of D allowed for the isolation of long-range integration (first and second-order) mechanisms. When $D > 1$, the contour could be detected by using element density information because the contour elements were closer to each other than the noise elements (Fig. 2a). However, when $D \leq 1$, this cue was not available and it was impossible to detect the contour without orientation specific long-range interactions (Fig. 2b). As the value of D decreased the strength of lateral connections increased. The actual strength of the long-range interactions in each subject could be defined by the value of D of the last correctly recognized card (D_{min} = threshold). On the color-defined cards the contour and the background were made up of colored patches instead of the Gabor stimuli. The luminance contrast and the size of the colored patches were randomized to ensure that the contour was purely defined by chromatic contrast and not by lumi-

nance contrast. Thirteen percent of all dots were red and 87% were green across the cards. The contour locations were equivalent to those of the orientation-defined cards. The difficulty level of the color set was matched exactly with the corresponding orientation-defined set on 156 adult subjects. Thus, this procedure provided us with essentially the same task demands for both the orientation and the color-defined cards.

The contours on the cards could not be detected purely by local filters or by neurons with large receptive field sizes corresponding to the size of the contour. The path of the contour could only be found by the integration of local orientation measurements. The noise forced the subject to do these local measurements at the scale of the individual Gabor signals, and to rely solely on long-range interactions between local filters while connecting the signals perceptually. Luminance information did not play a role in either the color- or the orientation-defined contour-detection cards. All visual cues were removed except for the long-range correlation among oriented elements. Therefore, the cards were supposed to isolate the long-range spatial interactions of low-level vision.

We used a battery of 10 orientation-defined contour integration cards, developed earlier by Kovács, Polat and Norcia (1996a), in the "main study". The D of the cards ranged between 1.1-0.65. In the "learning" and "spatial range" studies, new sets of 15 cards with increased range of D were generated, where the value of D varied between 1.2-0.5 in each. The value of D also ranged between 1.2-0.5 in the color-defined set.

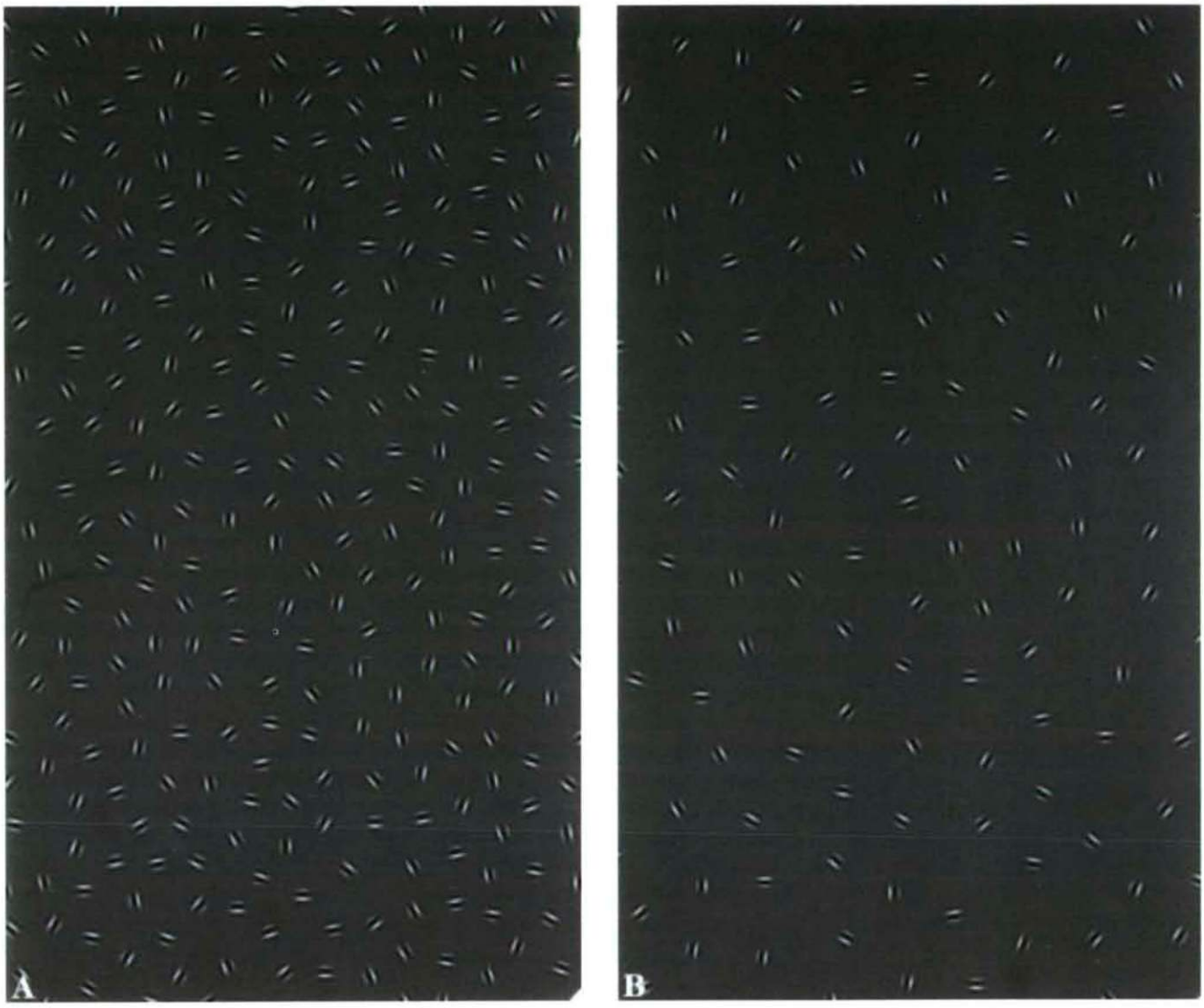


Figure 2. Two samples of the orientation-defined cards with **A.** a small (D = 0.65) and **B.** a high (D = 1.1) D value.

Procedure

The subjects were tested by using contour detection paradigm (Field et al. 1993; Kovács and Julesz 1993). The card version of the task was chosen, because it was found to be more suitable to test a large number of young children. Prior to the contour detection task the visual acuity of the children had been tested with E cards or Snellen cards depending on their age, and their stereovision with Randot test. Those with visual disorder, *e.g.* strabismus and amblyopia (2-5 subjects in each age group; approximately 18 subjects in the entire sample) and those with lack of compliance (5 in the entire sample) had been excluded from the study. All included subjects had normal or corrected-to-normal visual acuity. The two eyes of the subjects were tested separately, by testing the right eye first which further excluded subjects with a possibility of amblyopia (contour detection performance might be impaired and imbalanced in the two eyes of amblyopes (Kovács et al. 1996a; Hess et al. 1997; Pennefather et al. 1999). In the course of the "learning" and "spatial range" studies binocular presentation of the cards was applied. In the contour detection task the subjects' task was to identify the location of the contour and to trace the contour with their finger. Subjects were not forced to guess if they could not find the contour. The subjects were tested from a distance of about 0.50 m. The cards were presented in an increasing order of difficulty using a staircase method. One suprathreshold card was used as an explanatory example of the task before the test. We determined D_{min} in one session for each subject.

In our "main study" 510 subjects (413 children and 97 adults — 219 males, 291 females) were examined. The children ranged in age from 5 to 14 years in 5 different age groups (5-6, n=88; 6-7, n=98; 9-10, n=75; 10-11, n=64; 13-14, n=88). The group of 97 adults aged 19-30 years served as control. Subjects had been recruited by advertisement, and the experiments were carried out at daycare centers, schools, and colleges of Szeged, Hungary.

In the learning study we employed a training paradigm and determined D_{min} in a group of 60 adults (19-35 years) and in 60 (5-6 years) children on three consecutive days. We tested whether learning was specific for the stimulus dimensions of orientation and color in the contour-detection task. Equal numbers of subjects were divided randomly into four groups ("orientation", "color", "color-to-orientation", "orientation-to-color"). Both orientation- and color-defined cards were used depending on@the groups. The "orientation" group was tested with orientation-defined cards while the "color" group with color-defined cards on three consecutive days. Before testing with the color-defined cards the color vision of the subjects was assessed by using Ishihara plates. We tested whether the improvement transferred from color to orientation on a group of subjects that practiced with color-defined cards for two days and with orientation-defined cards on the third day ("color to orientation" group). The fourth group of subjects practiced with the orientation-defined cards for two consecutive days and was examined with the color cards on the third day ("orientation to color" group). The eye-specificity of learning was studied with orientation-defined cards on a different group of 10 (5-6 years) children by practicing the right eye on two consecutive days, and testing the left eye on the third day.

In the third "spatial range study" we investigated the spatial range of long-range horizontal interactions. Only orientation-defined cards were presented. One set with increased ($l = 9$; l is the wavelength of the Gabor patch), one with decreased ($l = 4.5$) and another with the original ($l = 7$) spacing of contour elements was used, while keeping the relative noise level constant. We tested 54 naive adults (19-30 years) and 30 naive children (5-6 years) with the three sets of cards in one session. In order to eliminate the effect of practice, we used a counterbalanced design for the order of presentation of the three sets. Statistical analysis was performed by using a two-tailed *t*-test. The performance of the different groups was compared to one another in each study.

Effect of late strabismus surgery in children with acquired esotropia

Subjects

We examined 10 (6 female and 4 male) 5-6 year old preschool children with acquired esotropia. Children were admitted to the Department of Ophthalmology, University of Szeged, Szeged, Hungary, for late strabismic surgery (strabismic group). The late surgery of the children was due to late referrals or ongoing treatment of amblyopia. The children received conservative therapy until there was no further improvement in their visual acuity. Therefore most of them had a visual acuity better than 20/40 (mild amblyopia; Kushner 1988) at the time of surgery (we will refer to the former amblyopic eye also as amblyopic). Three children were excluded from this study due to a history of prematurity, strabismus surgery and other ophthalmological diseases. Seven healthy, age-matched children with visual acuity of 1.0 (20/20) or better and normal binocular vision served as controls (control group).

Methods

During orthoptic examination the visual acuity of children was assessed using E cards. Their stereoacuity was obtained by Randot test and the angle of strabismus was measured by alternate prism and cover test by fixating to a light source at distance (5 m), and to a small object at near (0.33 m). Subsequently, visual evoked potentials to pattern reversal stimulation were recorded. Orthoptic examination and recordings were performed one day before and at least 3 months after surgery. Following surgery the degree of

binocularity was also assessed by synoptophore. Clinical data obtained by synoptophore were correlated with VEP amplitude values following surgery.

VEPs were recorded from an active scalp electrode placed midoccipitally (O_z in the 10-20 system). The system reference electrode was located on the left earlobe. The impedance was kept under 5 kW. Single channel recording was used. Subjects were seated on a height-adjustable revolving chair with arm- and headrest one meter from the monitor in a well-insulated darkened room with no windows. A fixation point was used and the attention of the children was drawn and kept by tales during the one hour session. Pattern reversal checkerboards with 80', 40', 20' check-sizes were used as stimulus for testing. The entire stimulus field subtended 15.64 degrees by 11.31 degrees of visual angle. The frequency of the stimulation was 1.8 Hz. The subjects wore appropriate correction for refractive errors. Monocular and binocular stimulation was performed. The amplitude and latency values of the P100 component were measured and one hundred responses were averaged. Further data of the strabismic group is indicated in Table 1. Statistical analysis was performed using three-way ANOVA and Student's *t*-test. We compared eyes, check-sizes, and timing within and between the strabismic and control groups.

Results

Maturation of visual spatial integration in children

In our "main study" we found a significant deficit in the spatial integration ability of 5-14 year old children compared to adults, using a contour detection paradigm (Fig. 3; Kozma et al. 1997). Five-six year old children could recognize the contour when the D value of the card equaled to or was less than 0.9 ($D_{min} < 0.9$; $D_{min} = 0.84$). As the value of D decreased, the performance of the children also decreased. Children missed the contours on about half of the cards. When older children were examined the performance got better. Children in the 13-14 year old group were able to see most of the contours (D min < 0.7; $D_{min} = 0.7$) and had similar performance to adults. Although the largest improvement seemed to occur between the 5-6 and 6-7 year old (D_{min} = 0.79) groups, there was a tendency for gradually increasing performance in the other age groups as well: in the 9-10 year old group $D_{min} = 0.76$, in the 10-11 year old group D_{min} = 0.72. The difference in contour-integration performance between the 5-6 and 13-14 year-age groups is significant (two-tailed *t*-test: $p < 0.005$). There is a slight improvement even after adolescence: $D_{min} = 0.67$ in the 19-30 year old group. There was no significant effect of eye and gender with respect to contour-detection performance ($p > 0.05$).

We conducted two further experiments (see below) to determine whether the significant age-effect was purely due to the maturation of the primary visual cortex (lower-level visual factors) or to higher-level cognitive developmental (such as search strategies) or motivational factors (such as perseverance in completing a difficult task).

Cue-specific learning in the contour integration task

In the "orientation" group tested with the new set of orientation-defined cards on consecutive days, we found significantly improved performance by the third day of practice. The improvement was more obvious in children (1st and 3rd day performances compared: two-tailed *t*-test: $p < 0.01$) than in adults ($p < 0.05$). Learning in the "color" group was similar to learning in the "orientation" group both in children (1st and 3rd day performances compared: $p < 0.01$) and in adults ($p < 0.05$). We found that experience with color cards did not significantly improve performance with orientation-defined cards, and vice versa.

Thus, learning seemed specific for the cue used for practice. It meant that there was no substantial transfer across the different visual cues of color and orientation. The transfer was completely absent in adults in both the "color to orien-

Table 1. Summary data of children

onset of strabismus		1.5 months-4 years
type of operation	recession	9 cases
	resection	0 "
	both	1 "
number of eyes operated on	one eye	3 cases
	both eyes	7 "

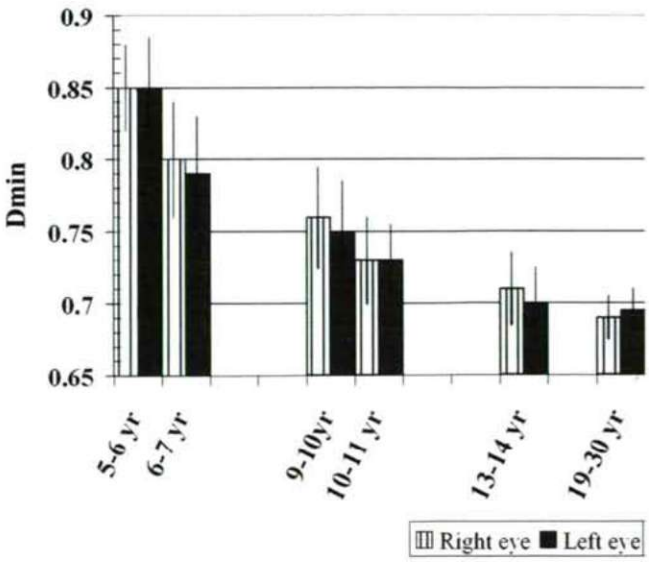


Figure 3. Contour-integration performance of 5-14 year old children and adults

tation" and the "orientation to color" groups. There was a slight but not significant tendency for transfer in children in the "color to orientation" group (1st day "orientation" and 3rd day "color to orientation" performances were not significantly different: $p = 0.073$; 3rd day "orientation" and 3rd day "color to orientation" performances were significantly different: $p < 0.05$). While testing transfer across eyes we found complete interocular transfer $D_{min} = 0.70$ on the third day of practice.

Spatial range of interactions in children and in adults

When testing with smaller contour spacing the performance of the children was better. At 9 λ contour spacing, performance was poorer than at 4.5 λ ($p < 0.01$, two-tailed *t*-test), or at 7 λ ($p < 0.01$). Unlike in children, D_{min} in adults is independent of contour spacing. The difference between children and adults in contour detection performance is bigger at larger contour spacing.

Effect of late strabismus surgery in children with acquired esotropia

The data of 10 children with treated strabismic amblyopia were analyzed. The mean corrected monocular visual acuity was 0.935. Six children had a monocular visual acuity of 1.0 for both eyes preoperatively. In one child the difference between the visual acuity in the amblyopic and fellow eye was two lines and in three cases it was only one line on the Snellen eye chart. Occlusion had to be reinstituted in one child because of an impairment in visual acuity in all other cases no changes were detected. The orthoptic findings of the children are indicated in detail in Table 2.

Prior to the operation, the mean angle of strabismus was 14.25 degrees at distance and 21.45 degrees at near. Preoperatively, the pattern evoked potential amplitude of the fellow eye was consistently higher than that of the amblyopic eye, but the binocular response was generally similar to that observed when stimulating the non amblyopic fellow eye.

The largest response was found on binocular stimulation (Kozma et al. 2001). Significant interocular differences were not detected in the control group (*t*-test: 80': $p = 0.994$; 40': $p = 0.698$; 20': $p = 0.734$). Differences for the P100 amplitude between the amblyopic and the fellow eye were significant by ANOVA analysis in the strabismic group ($F = 4.553$; $df = 2,14$; $p < 0.05$). The largest response was usually recorded on stimulation with the 80' check-size. There was a tendency for slightly longer latency values in the amblyopic eyes compared to the fellow eyes, however, the difference was not statistically significant ($p > 0.05$).

Visual evoked potentials showed clear changes under the effect of the surgery (three-way ANOVA: $F = 4.903$; $df = 2,14$; $p < 0.05$). Postoperatively, the amplitude values of P100 component increased significantly ($p < 0.05$), while no significant changes were seen in the latencies ($p > 0.05$; Fig. 4).

The most prominent changes were observed upon binocular stimulation at 80' and 40' checksize. Binocular fusion measured by synoptophore improved similarly after surgery, however, no correlation was found when comparing postoperative electrophysiological data on binocular stimulation and the synoptophore findings (Table 3).

Statistical analysis also showed a significant increase in VEP amplitude at 80' and 40' check-size for both the amblyopic and the fellow eyes (main effect of surgery: 80': $F = 4.839$; $df = 2,14$; 40': $F = 5.453$; $df = 2,18$; relationship between eyes postoperatively: $F = 16.565$; $df = 2,18$; interaction between eyes and check-size postoperatively: $F = 2.986$; $df = 4,36$; $p < 0.01$).

There were significant differences between the P100 amplitudes of the strabismic and the control group in relationship to check-size and eye (eyes: $F = 153.029$; $df = 5,20$; $p < 0.001$; between eyes and check-size: $F = 2.595$; $df = 10,40$; $p < 0.05$). Although amplitude values of the strabismic group were larger following the intervention, the differences observed between strabismics and controls were still significant (eyes: $F = 51.581$; $df = 5,30$; $p < 0.001$; between eyes and check-size: $F = 4.007$; $df = 10,60$; $p < 0.001$).

Table 2. Orthoptic findings of children before and after surgery

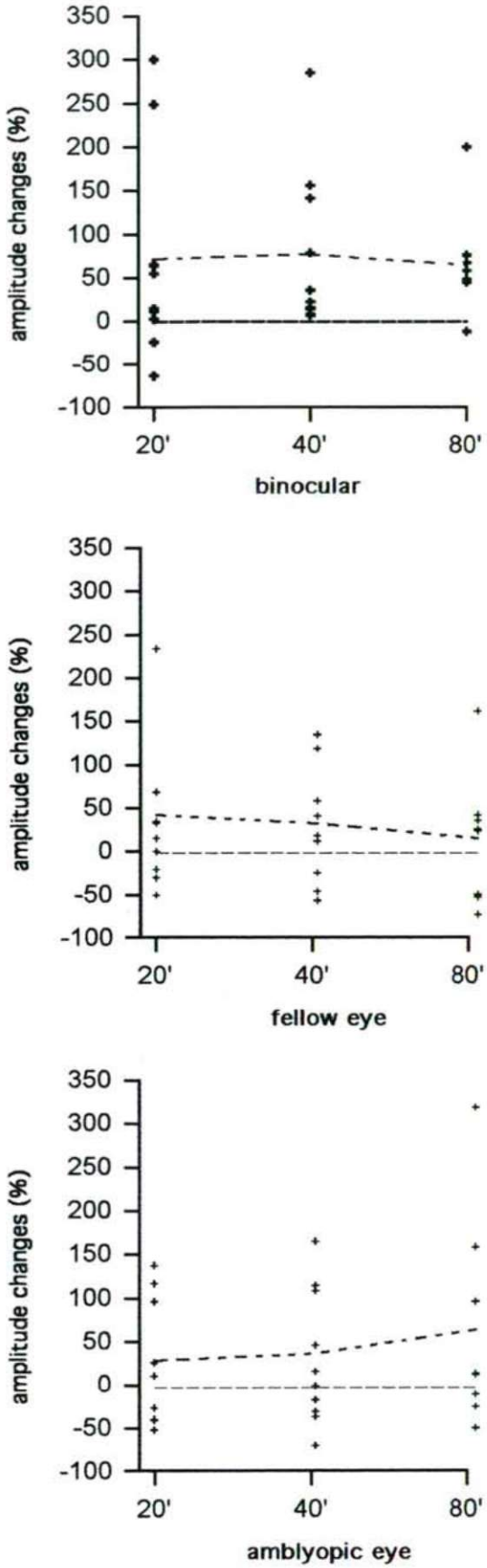
corrected monocular visual acuity	before surgery		0.6-1.0
	after surgery		0.25-1.0
angle of strabismus	before surgery	D	4-22°
		N	15-25°
	after surgery	D	0-7°
		N	3-6°
stereopsis	before surgery		-
	after surgery		-

D: at distance, N: at near

Discussion

Maturation of visual spatial integration in children

Classically, visual development has been assumed to be complete early in life to give way to cognitive development after the basic visual functions are established in infancy. Although behavioral studies of human visual development beyond the second year of age are rare, there is indication that children may have problems in tasks involving integration of information across the visual field (visual segmentation and form identification based on texture; Atkinson and Braddick 1992; Sireteanu and Rieth 1993), motion (Hollants-Gilhuijs 1998a) or color-contrast (Hollants-Gilhuijs 1998b); recognition of incomplete objects; Gollin 1960). Our result also confirms the late maturation of a visual function: contour detection performance shows significant development in



children between ages 5-14 years. The results indicate a significant age-effect, but there is no significant difference between eyes and gender.

It has been shown that low-level visual tasks significantly improve with practice (*e.g.* hyperacuity; Poggio et al. 1992), visual discrimination (Fiorentini and Berardi 1980), pop-out (Karni and Sagi 1993), and visual search tasks; Sireteanu and Rettenbach 1995) and seem to be specific for stimulus parameters and some of them even for retinal location. Therefore, we tested the contribution of low-level visual mechanisms to our developmental effect by employing a learning paradigm in children and in adults ("learning study"). We found significant improvement with practice and the improvement was specific for the visual cue defining the task in both age groups similar to other low-level visual tasks. Our findings demonstrate that the strength of interactions can be extended even with a short training in children with normal vision; however, a relatively long consolidation period might be necessary for such interactions to improve (Hollants-Gilhuijs 1998a).

The slight but not significant tendency for transfer in children in the "color to orientation" group might indicate that cognitive/motivational factors cannot be excluded completely in children, but do not explain our data. The similar trends found in adults and children imply that the same mechanisms might be responsible for the performance in both groups. A high degree of stimulus specificity usually suggests that the plastic neuronal changes of learning took place at early cortical levels where the basic stimulus dimensions are still separable. The transfer found across eyes indicates that learning must have occurred in the cortex at a level where information from the two eyes is combined, and the contribution from subcortical structures is insubstantial. Consequently, our result implies that the reduced contour integration performance of 5-14 year old children is a low-level perceptual effect and cannot be explained by high-level cognitive developmental factors or by non-visual factors.

As mentioned before, long-range interactions might subserve the integration process (Mitchinson and Crick 1982; Nelson and Frost 1985; Ts'o and Gilbert 1988; Gilbert 1998). The possible anatomical substrate of the long-range interactions is the intrinsic horizontal connections of the primary visual cortex (Rockland and Lund 1982; Gilbert and Wiesel 1983). The plasticity of these interactions was demonstrated in psychophysical studies where the spatial range of the interactions was extended in adult human subjects (Polat and Sagi 1994). Therefore, we assumed that the analysis of the actual spatial ranges of interactions in children and in adults might give some explanation of the developmental effect. In the "spatial range study" we found that contour integration

Figure 4. Amplitude values under the effect of surgery

performance depends on absolute contour spacing in children and not on the level of noise or signal-to-noise ratio *per se*. On the contrary, the performance of adults in the tested range is limited only by display parameters (signal-to-noise ratio) and not by the absolute range of cortical interactions (the range of contour spacing that can be tested at all is limited: above 9 λ spacing the number of contour elements would be too small to provide comparable conditions). The results suggest that long-range spatial interactions — although probably present at an early age — might not be functioning at an adult level in terms of their spatial range.

Let us mention that, in addition to these lateral connections, higher level processing such as modulatory feedback connections of extrastriate origin and/or participation of "intermediate" level cortical areas (V4) might also play some role (Kovács and Julesz 1993; Wilson and Wilkinson 1998). High-level perceptual interpretations function to make sense of the stimulus and it might well be that young children cannot generate and apply these interpretations that easily. Interestingly, Callaway and Katz (1990) indicated a delayed postnatal development of feedback connections between V2 and V1 in humans. More recent studies raise the possibility of a significant increase in the number of cortical cells between birth and six years of age (Shankle et al. 1998a), showing an extended structural maturation of the human cortex, including the early visual areas (Shankle et al. 1998b).

In summary, we found significant improvement in children between ages 5 to 14 years in visual spatial integration using a contour-detection task. We hypothesize that long-range spatial interactions might have a shorter spatial range in children than in adults since search deficiency (Sireteanu and Rettenbach 1996b) should result in an opposite tendency (improving performance with decreasing number of distractors). Learning can enhance the span of these interactions. Cue-specific learning indicates the involvement of fairly low-level perceptual mechanisms. Therefore, the observed lag in children is probably truly perceptual because motivational factors or cognitive mechanisms would not be expected to generate different tendencies among children and adults in terms of contour spacing. Our results are in accordance with recent anatomical (Burkhalter et al. 1993a) and psychophysical (Atkinson and Braddick 1992; Sireteanu and Rieth 1993) findings and indicate that the orientation-based segmentation carried out by long-range spatial interactions may have an extended maturational period (Kovács et al. 1999).

The long-range connections of the visual cortex are also assumed to take part in contextual effects in perception (Gilbert and Wiesel 1983; Ts'o and Gilbert 1988; Kovács and Julesz 1993, 1994; Kovács 1996b; Gilbert 1998). There is behavioral evidence that these contextual effects might be largely mediated by the occipitotemporal (or ventral) visual stream (Goodale and Haffenden 1998a; Goodale and Humphrey 1998b; Haffenden and Goodale 1998; Marotta et al. 1998). Thus, our result further suggests the different maturational rate of the two major subsystems of vision, namely, the dorsal (occipitoparietal) stream and the ventral (occipitotemporal) stream that are going to be discussed in more details in the Discussion section.

Effect of late strabismus surgery in children with acquired esotropia

The proper timing of surgery and its functional consequences in acquired esotropia, unlike infantile esotropia, have not been studied extensively because those suffering from it have a better chance for functional recovery (Birch et al. 1990).

Leguire et al. (1991) studied pre- and postoperative binocular summation of pattern visual evoked response in 9 early onset esotropic (1-58 months old) children. They found a significant difference between binocular and monocular amplitudes in accordance with 64% increase in the binocular and a 28% decrease in the monocular amplitude postoperatively. We studied 5-6 year old children with acquired esotropia several years beyond the end of the classical critical period. Our results also showed a significant improvement of the amplitude of pattern-visual evoked potentials after surgical intervention. The most consistent increase was found in the amplitude values of the P100 component to binocular stimulation especially with a moderately large check-size (40'). In the view of the classical developmental theory this considerable increase of evoked potentials in children

Table 3. Comparison of electrophysiological and synoptophore data

binocular interaction on VEP	before surgery	binocular summation	6 cases
		binocular facilitation	1 "
		binocular inhibition	3 "
	after surgery	binocular summation	10 "
		binocular facilitation	0 "
		binocular inhibitoion	0 "
synoptophore	after surgery	HRC	3 cases
		NHRC	3 "
		CS	4 "

(Summation index introduced by Apkarian et al. (Apkarian et al. 1981b) and modified by Nuzzi and Franchi (1983): HRC: Harmonic Retinal Correspondence, NHRC: Non-harmonic Retinal Correspondence, CS: peripheral fusion with central suppression (von Noorden 1985)

following corrective surgery for acquired strabismus was an unexpected finding. Similarly good binocular fusion was observed in 6 cases (60%) and peripheral fusion in 4 cases (40%) by synoptophore following surgery.

Unlike Amigo et al. (1978) we observed no correlation between the strength of binocular summation (Apkarian et al. 1981b; Nuzzi and Franchi 1983) on the VEP and the degree of binocularity obtained by clinical evaluating method after surgery (Table 3). Besides, Amigo et al. (1978) noted that binocular VEP summation in stereodeficient adults is extremely variable. Postoperatively we noticed VEP summation in most cases and found no facilitation for reversal stimulation in amblyopes which is in agreement with the findings of Shawkat and Kriss (1997), but contrary to the findings of Apkarian et al. (1981a). Shawkat and Kriss (1997) had found that reversal P100 component showed the greatest difference between normals and amblyopes for small (12') and moderate sized checks (20', 50'). Similarly, we also observed the most pronounced binocular enhancement for the moderate check-size. Our finding supports earlier results (Nuzzi and Franchi 1983, Shea et al. 1987) despite of slight differences in the different studies.

Achievement of fusion (O'Keefe et al. 1996; Willshaw and Keenan 1991) is the main goal of strabismus surgery both in children and in adults (Hohmann and Creutzfeld 1975; Kraft 1998). Fusion is thought to be an acquired reflex (Aslin 1977; Starger and Birch 1986), hence can be regained when it is lost. Previous fusional ability present before the onset of strabismus can probably be reestablished by surgery involving at least some binocular neurons in the visual cortex (Hohmann and Creutzfeld 1975; Morris et al. 1993; Wright 1996). The marked postoperative changes in the visual evoked potential to binocular stimulation in the strabismic group therefore are probably due to the attainment of fusional mechanisms. Patients with acquired esotropia were shown to have a better chance for binocularity than patients with infantile esotropia (Kushner 1994). Even adults with no previous alignment have the potential for peripheral fusion after strabismus surgery (Morris et al. 1993). Alignment of the eyes and binocular fusion is the prequisite of stereopsis, althought it also largely depends on visual acuity (Daw 1995). Reduction in acuity can lead to degraded stereopsis even in normal people. Unfortunately there is no chance to regain it when it was impaired within the first 18 months (Sloper and Collins 1998). Our subjects had no detectable stereopsis neither before nor after operation, thus probably lost their stereopsis within its critical period and could not regain it.

The reduced amplitude values of the amblyopic eye compared to the fellow eye can be due to the stronger synchronization of the neurons' responses driven by the normal than by the amblyopic eye (Roelfsema et al. 1994). The significantly reduced amplitude values of the fellow eyes even with good visual acuity in the amblyop group can probably be explained by the presurgical occlusion therapy applied to all children (Arden and Barnard 1979; Barnard and Arden 1979; Wilcox and Sokol 1980; Leguire et al. 1995; Shawkat et al. 1998). It appears that the amblyopic eye influences the fellow eye through interocular interactions (Sclar et al. 1986; Wali et al. 1991) Our finding is in agreement with that of previous groups, and indicates that despite of good visual acuity (20/20) the fellow eyes of the amblyopes are not functionally normal (Kandel et al. 1976,1980; Rentschler and Hilz 1979; Leguire et al. 1990).

The postoperative improvement of amplitude values of both amblyopic and fellow eyes observed during the 3-month follow up cannot be attributed to physiological rate of cortical maturation in these children (see the introductory paragraphs). Although the role of discontinuation of occlusion therapy, increase of visual field (Bowering et al. 1997; Hohmann and Creutzfeld 1975; Worthamn and Greenwald 1989; Kushner 1994), changes in the organization of afferent inputs as well as in the intracortical interactions (Konig et al. 1993) cannot be excluded entirely.

In conclusion, our results provide evidence about enhancement in binocular function after strabismus surgery, even if performed years beyond the end of the classical critical period. Since no correlation was determined between the amount of improvement in the VEP and in the synoptophore findings, the VEP results could also reflect the epiphenominal nature of our finding besides improved binocular function. However, good ocular alignment itself can also enhance the signals coming from the two eyes without any substantial changes in the brain.

Conclusions

Much of our knowledge of the external world is gained through the visual system. As we have seen in the previous sections the visual system is immature at birth and is relatively mutable and plastic as it develops in infancy (Fiorentini 1984; Daw 1994). Looking for timing is relevant since the onset of critical periods and duration of plasticity for different visual functions in humans are not understood precisely. Some begin by few months of age and some may last 5 or 7 years (Banks and Aslin 1975; Marg et al. 1976; Huttenlocher et al. 1982) or continues through puberty (Vaegan and Taylor 1979; Daw 1994, 1995). Hormonal changes around puberty might determine the end of plasticity (Daw et al. 1991). The development of visual functions usually correlates well with the morphological changes detected in the visual cortex. In the face of the uncertainty concerning the onset and length of the critical periods and plasticity, it is very timely and worthwhile to study various visual functions in extended populations but in more specific age groups.

Both of our studies were unique of their kind and both focused on the maturation and plasticity of visual functions in children after the age of 5 years. We found significant

changes in a visual function through adolescence in our spatial integration study. The improvement in spatial integration with practice and with age was considered to be due to plasticity and at least functional immaturity of horizontal connections in the visual cortex. We similarly noticed significant functional changes after surgery beyond the end of the classical critical period in the amblyopic or formerly amblyopic visual system in our second study. This also provides evidence for the plasticity of visual functions years beyond infancy.

Based upon our results the question arises why researchers and clinicians did have a theory of a shorter span (by 2 years of age) of maturation of visual functions? The answer to this question is not easy since the whole picture of development is not yet known. The different methodology, the bad cooperation of children at certain ages and the distinct explanation of definitions clearly played an important role. Due to scientific and methodological development the interpretation of different definitions has also undergone some changes. Hence, what previously had been considered to be the critical period seems to be only the first part of it. The visual system, however, could retain plasticity for a longer time and this involves a prolonged susceptibility to abnormal stimuli and responsiveness to treatment. The second reason why these phenomena of late visual maturation have not been described could be the historical fact that the classical description of the visual development in children had been completed before the discovery of the parallel visual streams. Thus, no special attention has been paid to the reinvestigation of all visual developmental phenomena. As also mentioned before, we suppose that our findings can be further related to the different maturational time frame of the two parallel visual pathways, namely, the dorsal and the ventral streams (Ungerleider and Mishkin 1982) that subserve different aspects of vision.

The occipitotemporal or ventral stream is involved in more refined perceptual categorization, object recognition, and mediates contextual effects. The occipitoparietal or dorsal stream is concerned with the online control of goal directed actions (Ungerleider and Mishkin 1982; Goodale and Milner 1992; Milner and Goodale 1995). The color sensitive parvo- (P) and the motion and luminance sensitive magnocellular (M) retinocortical pathways (Livingstone and Hubel 1988) do not project separately to the ventral and the dorsal streams (Livingstone and Hubel 1988; Shapley 1990; Wright 1995). It is suggested, that the ventral and dorsal streams both receive inputs from the M and P pathways, although most of the input to the dorsal stream is magno in origin (Milner and Goodale 1995). The ventral pathway gets at least as much input from the magno as it does from the parvo system (Stoner and Albright 1993; Milner and Goodale 1995; Sawatari and Callaway 1996; Neville and Bavelier 2000).

Concerning their developmental pattern, not many studies are available and the findings are controversial. Global models of visual development have occasionally been proposed in which the P pathway precedes the M pathway in development or vice versa (Teller 2000). It is very likely that each pathway and their function develops at different rates. There is little, if any, neuroanatomical evidence in humans about the time span of the development of the two streams. According to anatomical studies in macaque monkeys, the dorsal pathway matures earlier than the ventral stream (Bachevalier et al. 1991, Distler et al. 1996). Neville and Bavelier (2000), on the other hand, suggest that the dorsal visual pathway has a more prolonged maturational time course.

Dobkins et al. (1999) and Teller (2000), in accordance with others (LeVay et al. 1980, Livingstone and Hubel 1988) propose a precocious development of the M pathway according to their human psychophysical findings. Tassinari et al. (1994) also provide evidence for a later development of the M fibers of the optic tract compared to the P-fibers. The larger cells in the M-layers of the corpus geniculatum laterale also have a longer period of susceptibility than the small cells in the P-layers (Hickey 1977; Wiesel 1982). The rapid growth ends at around 6 months for the small cell type and at around 12 months for the larger cell type (Hickey 1977).

Concerning the maturation of the two visual streams in amblyopia, it is widely accepted that amblyopes have impaired form vision that is strongly related to the P system. Abnormal P-cell development, however, was found predominantly only in cases of pattern deprivation amblyopia, while M-neuron maldevelopment was reported mainly in cases of strabismus or severe monocular blur. Maldevelopment of M-cells might also explain motor abnormalities in amblyopia (Wright 1995). Kiorpes et al. (1996b) found some deficit in the middle temporal area of the visual system of the amblyopic monkeys, that is part of the dorsal stream. Thus, the situation is not quite clear in amblyopia, either.

What can explain the extended time frame of plasticity concerning the amblyopic visual system? It was found that amblyopia only slows down the development and causes relative immaturity (Kiorpes 1992b; Kiorpes and Movshon 1996a). Thus, it is not surprising that the visual system is more plastic, and remains plastic for a prolonged period of time when amblyopia is present (Ciuffreda et al. 1991). The findings of Kiorpes et al. (1996b) and the notion that the VEP latency is considerably less prolonged in amblyopes than the reaction time (the time required to produce a motor response to a visual stimulus; von Noorden 1961) suggest that higher levels of the visual system are probably also involved in amblyopia. As we have seen earlier, higher levels keep plasticity for a longer span than lower levels (Daw 1995). It is probable that these factors might all contribute to the prolonged plasticity in amblyopes to some extent.

In conclusion, contrary to the theory that visual functions mature by 2 years of age, we have shown that the maturation of at least some visual functions and the plasticity of the visual cortex last much longer than it was indicated before. This notion has a considerable theoretical importance and practical benefit in clinical efforts aiming at the enhancement of visual abilities in children. It raises the possibility of functional improvement at a later age and promotes the trials for treatment up to the puberty or even later.

Acknowledgments

We wish to thank Drs. Andrea Deák and Márta Janáky for their help and advice. We are further grateful to Dr. Krisztina Boda for her assistance in statistical analysis. This work was supported by Soros Grants: 222/3588, 222/3/3745, by the J.S. McDonnel Foundation Grant 9560 and by the Hungarian National Research Found (OTKA, Hungary) Grants 016959, 29817 as well as the ETT Grant (Hungary) 57404.

References

Abramov I, Gordon J, Hendrickson A, Hainline L, Dobson V, LaBossiere E (1982) The retina of the newborn human infant. Science 217:265-267.

Amigo G, Fiorentini A, Picchio M, Spinelli D (1978) Binocular vision tested with visual evoked potentials in children and infants. Invest Ophthalmol Vis Sci 17:910-915.

Apkarian P, Levi D, Tyler CW (1981a) Binocular facilitation in the visual-evoked potential of strabismic amblyopes. Am J Optom 58:820-830.

Apkarian PA, Nakayama K, Tyler CW (1981b) Binocularity in the human visual evoked potentials: facilitation, summation and suppression. Electroencephalogr Clin Neurophysiol 51:32-48.

Arden GB, Barnard WM (1979) Effect of occlusion on the visual evoked response in amblyopia. Trans Ophthalmol Soc UK 99:419-426.

Arden GB, Barnard WM, Mushin AS (1974) Visually evoked responses in amblyopia. Br J Ophthalmol 58:183-194.

Arthur BW, Smith JT, Scott WE (1989) Long-term stability of alignment in the monofixation syndrome. J Pediatr Ophthalmol Strabismus 26:224-231.

Asbury T, Burke MJ (1995) Strabismus. In Vaughan DG, Asbury T, Riodan-Eva P, eds., General Ophthalmology. 14th ed., Appleton & Lange, Norwalk, CT, 226-244.

Aslin RN (1977) Development of binocular fixation in human infants. J Exp Child Psychol 23:133-150.

Atkinson J, Braddick O (1992) Visual segmentation of oriented textures by infants. Behav Brain Res 49:123-131.

Atkinson J (1984) Human visual development over the first 6 months of life. A review and a hypothesis. Hum Neurobiol 3:61-74.

Atkinson J, French J, Braddick O (1981) Contrast sensitivity function of preschool children. Br J Ophthalmol 65:525-529.

Atkinson J (1979) Development of optokinetic nystagmus in the human infant and monkey infant: An analogue to development in kittens. In Freeman RD, ed., Developmental Neurobiology of Vision. Plenum Publishing Corp., New York.

Bachevalier J, Hagger C, Mishkin M (1991) Functional maturation of the occipitotemporal pathway in infant rhesus monkeys. In Lassen NA, Ingvar DH, Raichle ME, Friberg L, eds., Brain Work and Mental Activity. Alfred Benzon Symposium. Vol. 31. Munksgaard, Copenhagen, 231-240.

Banks MS, Aslin RN (1975) Sensitive period for the development of human binocular vision. Science 190:675-677.

Barlow HB (1975) Visual experience and cortical development. Nature 258:199-204.

Barnard WM, Arden GB (1979) Changes in the visual evoked response during and after occlusion therapy for amblyopia. Child Care Health Dev 5:421-430.

Barten S, Birns B, Ronch J (1971) Individual differences in the visual pursuit behavior of neonates. Child Dev 42:313-319.

Beazley LD, Illingworth DJ, Jahn A, Greer DV (1980) Contrast sensitivity in children and adults. Br J Ophthalmol 64:863-866.

Birch EE, Stager DR, Berry P, Everett ME (1990) Prospective assessment of acuity and stereopsis in amblyopic infantile esotropes following early surgery. Invest Ophthalmol Vis Sci 31:758-765.

Birch EE, Gwiazda J, Bauer JA, Naegele J, Held R (1983) Visual acuity and its meridional variations in children aged 7-60 months. Vision Res 23:1019-1024.

Birch EE, Gwiazda J, Held R (1982) Stereoacuity development of crossed and uncrossed disparities in human infants. Vision Res 22:507-513.

Blomdahl S (1979) Ultrasonic measurements of the eye in the newborn infant. Acta Ophthalmol 57:1048-1056.

Bodis-Wollner I, Ghialardi MF, Mylin LH (1986) The importance of stimulus selection in VEP practice: the clinical relevance of visual physiology. In Cracco RQ, Bodis-Wollner I, eds., Frontiers of Clinical Neuroscience: Evoked Potentials. AR Liss, New York, 15-27.

Bodis-Wollner I, Barbis MC, Mylin LH, Julesz B, Kropfl W (1981) Binocular stimulation reveals cortical components of the human visual evoked potential. Electroencephalogr Clin Neurophysiol 52:298-305.

Bornstein MH, Kessen W, Weiskopf S (1976) The categories of hue in infancy. Science 191:201-202.

Bowering ER, Maurer D, Lewis TL, Brent HP (1997) Constriction of the visual field of children after early visual deprivation. J Pediatr Ophthalmol Strabismus 34:347-356.

Braddick OJ, Wattam-Bell J, Atkinson J (1986) Orientation-specific cortical responses develop in early infancy. Nature 320:617-619.

Braddick OJ, Atkinson J (1983) Some recent findings on the development of human binocularity: a review. Behav Brain Res 10:141-150.

Braddick OJ, Atkinson J, Julesz B, Kropfl W, Bodis-Wollner I, Raab E (1980) Cortical binocularity in infants. Nature 288:363-365.

Bradley A, Freeman RD (1982) Contrast sensitivity in children. Vision Res 22:953-959.

Bronson GW (1982) Structure, status and characteristics of the nervous system at birth. In Stratton P, ed., Psychobiology of the Human Newborn. Wiley, Chichester.

Bronson GW (1974) The postnatal growth of visual capacity. Child Dev 45:873-890.

Bruce V, Green PR, Georgeson MA (1996) Visual Perception. 3rd ed., Psychology Press, UK.

Burkhalter A, Bernardo KL, Charles V (1993a) Development of local circuits in human visual cortex. J Neurosci 13:1916-1931.

Burkhalter A (1993b) Development of forward and feed-back connections between areas V1 and V2 of human visual cortex. Cereb Cortex 3:476-486.

Burr DC, Morrone C, Fiorentini A (1996) Spatial and temporal properties of infant colour vision. In Vital-Durand F, Atkinson J, Braddick OJ, eds., Infant vision. 1st ed., Oxford University Press (EBBS Publication Series), New York, 63-78.

Callaway EM, Katz LC (1990) Emergence and refinement of clustered horizontal connections in cat striate cortex. J Neurosci 10:1134-1153.

Carr RE, Siegel IM (1982) Visual Electrodiagnostic Testing. A Practical Guide for the Clinician. Williams & Wilkins, New York.

Chugani HT, Phelps ME, Mazziotta JC (1987) Positron emission tomography study of human brain functional development. Ann Neurol 22:487-497.

Cibis L (1975) History of amblyopia and its treatment. Fifth annual Richard C. Scobee Memorial Lecture. Am Orthopt J 25:54-61.

Ciuffreda KJ, Levi DM, Selenow A (1991) Amblyopia. Butterworth-Heinemann, USA.

Ciuffreda KJ (1986) Visual system plasticity in human amblyopia. In Hilfer

SR, Sheffield JB, eds., Cell and Developmental Biology of the Eye: Development of Order in the Visual System. Springer Verlag, New York, 211-244.
Conel JL (1939-1967) The Postnatal Development of the Human Cerebral Cortex. Vols. I-VIII. Harvard University Press, Cambridge, MA.
Costenbader FD, Bair D, McPhail A (1948) Vision in strabismus: A preliminary report. Arch Ophthalmol 40:438-453.
Crognale MA, Kelly JP, Chang S, Weiss AH, Teller DY (1997) Development of pattern visual evoked potentials: longitudinal measurements in human infants. Optom Vis Sci 74:808-815.
Dakin SC, Hess RF (1998) Spatial-frequency tuning of visual contour integration. J Opt Soc Am A Opt Image Sci Vis 15:1486-1499.
Daw NW (1995) Visual Development. Plenum Press, New York, 123-163.
Daw NW (1994) Mechanisms of plasticity in the visual cortex. The Friedenwald Lecture. Invest Ophthalmol Vis Sci 35:4168-4179.
Daw NW, Sato H, Fox K, Carmichael T, Gingerich R (1991) Cortisol reduces plasticity in the kitten visual cortex. J Neurobiol 22:158-168.
Dayton GO, Jones MH (1964a) Analysis of characteristics of fixation reflex in infants by use of direct current electrooculography. Neurology 14:1152-1156.
Dayton Jr. GO, Jones MH, Steele B, Rose M (1964b) Developmental study of coordinated eye movements in the human infant. Arch Ophthalmol 71:871-875.
de Courten C, Garey LJ (1982) Morphology of the neurons in the human lateral geniculate nucleus and their normal development. Exp Brain Res 47:159-171.
Distler C, Bachevalier J, Kennedy C, Mishkin M, Ungerleider LG (1996) Functional development of the corticocortical pathway for motion analysis in the macaque monkey: A 14 C-2-deoxyglucose study. Cereb Cortex 6:184-195.
Dobkins KR, Anderson CM, Lia B (1999) Infant temporal contrast sensitivity functions (tCSFs) mature earlier for luminance than for chromatic stimuli: evidence for precocious magnocellular development? Vision Res 39:3223-3239.
Dobson V, Teller DY (1978) Visual acuity in human infants: a review and comparison of behavioral and electrophysiological studies. Vision Res 18:1469-1483.
Eggers HM (1993) Amblyopia. In Diamond GR, Eggers HM, eds., Strabismus and Pediatric Ophthalmology. Textbook of Ophthalmology. Vol. 5. Mosby, London, Chapter 13.
Epelbaum M, Milleret C, Buisseret P, Dufier JL (1993) The sensitive period for strabismic amblyopia in humans. Ophthalmology 100:323-327.
Eustis HS (1995) Postnatal development. In Wright KW, ed., Pediatric Ophthalmology and Strabismus. 1st ed., Mosby, USA, 45-57, 119-126.
Field DJ, Hayes A, Hess RF (1993) Contour integration by the human visual system: Evidence for a local "association field". Vision Res 33:173-197.
Fiorentini A, Trimarchi C (1992). Development of the temporal and nasal visual fields during infancy. Vision Res 32:1609-1621.
Fiorentini A (1984) Visual development. Hum Neurobiol 3:59-60.
Fiorentini A, Berardi N (1980) Perceptual learning specific for orientation and spatial frequency. Nature (London) 287:43-44.
Fishman GA, Sokol S (1990) Electrophysiologic Testing. 1st ed., Ophthalmic Monographs. American Academy of Ophthalmology, USA.
Fox R, Patterson R, Francis EL (1986) Stereoacuity in young children. Invest Ophthalmol Vis Sci 27:598-600.
Fox R, Aslin RN, Shea SL, Dumais ST (1980). Stereopsis in human infants. Science 207:323-324.
Friede RL, Hu KH (1967) Proximo-distal differences in myelin development in human optic fibres. Z Zellforsch 79:259-264.
Garey LJ (1984) Structural development of the visual system of man. Hum Neurobiol 3:75-80.
Garey LJ, de Courten C (1983) Structural development of the lateral geniculate nucleus and visual cortex in monkey and man. Behav Brain Res 10:3-13.
Giedd JN, Snell JW, Lange N, Rajapakse JC, Casey BJ, Kozuch PL, Vaituzis AC, Vauss YC, Hamburger SM, Kaysen D, Rapoport JL (1996) Quantitative magnetic resonance imaging of human brain development: ages 4-18. Cereb Cortex 6:551-560.
Gilbert CD (1998) Adult Cortical dynamics. Physiol Rev 78:467-485.
Gilbert CD, Wiesel TN (1983) Clustered intrinsic connections in cat visual cortex. J Neurosci 3:1116-1133.
Gollin ES (1960) Developmental studies of visual object recognition of incomplete objects. Percept Mot Skills 11:289-298.
Goodale MA, Haffenden A (1998a) Frames of reference for perception and action in the human visual system. Neurosci Biobehav Rev 22:161-172.
Goodale MA, Humphrey GK (1998b) The objects of action and perception. Cognition 67:181-207.
Goodale MA, Milner AD (1992) Separate visual pathway for perception and action. Trends Neurosci 15:20-25.
Gordon RA, Donzis PB (1985) Refractive development of the human eye. Arch Ophthalmol 103:785-789.
Gottlob I, Stangler-Zuschrott E (1990) Effect of levodopa on contrast sensitivity and scotomas in human amblyopia. Invest Ophthalmol Vis Sci 31:776-780.
Greenwald MJ, Parks MM (1990) Treatment of amblyopia. In Tasman W, Jaeger EA, eds., Duane's Clinical Ophthalmology. Vol.1. Lippincott, Philadelphia, 1-9.
Groffman S (1978) Psychological aspects of strabismus and amblyopia - a review of the literature. J Am Opt Assoc 49:995-999.
Gwiazda J, Brill S, Mohindra I, Held R (1980) Preferential looking acuity in infants from two to fifty-eight weeks of age. Am J Optom Physiol Opt 57:428-432.
Haffenden A, Goodale MA (1998) The effect of pictorial illusion on prehesion and perception. J Cogn Neurosci 10:122-136.
Halliday AM, Barrett G, Blumhardt LD, Kriss A (1979) The macular and paramacular subcomponents of the pattern evoked response. In Lehmann D, Callaway E, eds., Human Visual Evoked Potentials: Applications and Problems. Plenum Press, New York, 135-151.
Harding GFA, Grose J, Wilton A, Bissenden JG (1989) The pattern reversal VEP in short gestation infants. Electroencephalogr Clin Neurophysiol 74:76-80.
Harwerth RS, Smith EL III, Duncan GC, Crawford MLJ, von Noorden GK (1986) Multiple sensitive periods in the development of the primate visual system. Science 232:235-238.
Hecht KA, Straus H, Denny M, Taylor F, Garrett M (1996) Pediatric Ophthalmology and Strabismus. Basic and Clinical Science Course. American Academy of Ophthalmology, San Francisco, USA.
Held R, Birch EE, Gwiazda J (1980) Stereoacuity of human infants. Proc Natl Acad Sci USA 77:5572-5574.
Henc-Petrinovic L, Deban N, Gabric N, Petrinovic J (1993) Prognostic value of visual evoked responses in childhood amblyopia. Eur J Ophthalmol 3:114-120.
Hendrickson A, Yuodelis C (1984) The morphological development of the human fovea. Ophthalmology 91:603-612.
Hess RF, McIlhagga W, Field DJ (1997) Contour integration in strabismic amblyopia: the sufficiency of an explanation based on positional uncertainty. Vision Res 37:3145-3161.
Hess RF, Field DJ, Watt RJ (1990) The puzzle of amblyopia. In Blakemore C, ed., Vision Coding and Efficiency. Cambridge University Press, Cambridge, 267-279.
Hickey TL (1981) The developing visual system. Trends Neurosci 4:41-44.
Hickey TL (1977) Postnatal development of the human lateral geniculate nucleus: relationship to a critical period for the visual system. Science 198:836-838.
Hohmann A, Creutzfeldt OD (1975) Squint and the development of binocularity in humans. Nature 254:613-614.
Hollants-Gilhuijs MA, Ruijter JM, Spekreijse H (1998a) Visual half-field development in children: Detection of motion-defined forms. Vision Res 38:651-657.
Hollants-Gilhuijs MA, Ruijter JM, Spekreijse H (1998b) Visual half-field development in children: Detection of colour-contrast-defined forms. Vision Res 38:645-649.

Hubel D.H., Wiesel T.N (1970) The period of susceptibility to the physiological effects of unilateral eye closure in kittens. J Physiol (London) 206:419-436.

Huttenlocher PR, Dabholkar AS (1997) Regional differences in synaptogenesis in human cerebral cortex. J Comp Neurol 387:167-178.

Huttenlocher PR (1994) Synaptogenesis, synapse elimination, and neural plasticity in human cerebral cortex. In Nelson CA, ed., Threats to Optimal Development: Integrating Biological, Psychological, and Social Risk Factors. Vol. 27. Erlbaum, Hillsdale, NJ, 35-54.

Huttenlocher PR, de Courten C (1987) The development of synapses in striate cortex of man. Hum Neurobiol 6:1-9.

Huttenlocher PR, de Courten C, Garey LJ, van der Loos H (1982) Synaptogenesis in human visual cortex - evidence for synapse elimination during normal development. Neurosci Lett 33:247-252.

Isenberg SJ (1989) Visual development. In Isenberg SJ, ed., Eye in infancy. 1st ed., Year Book Med. Publication, Chicago, 40-56.

Johnson MH (1990) Cortical maturation and the development of visual attention in early infancy. J Cogn Neurosci 2:81-95.

Jones JP, Palmer LA (1987) An evaluation of the two-dimensional Gabor filter model of simple receptive fieldsin cat striate cortex. J. Neurophys 58:1233-1258.

Kandel GL, Grattan PE, Bedell HE (1980) Are the dominant eyes of amblyopes normal? Am J Optom Physiol Opt 57:1-6.

Kandel GL, Bedell HE, Fallon JH (1976) Near final scotopic thresholds in normal eyes and in the dominant eyes of amblyopes. Ophthalmic Res 8:425-433.

Karni A, Sagi D (1993) The time course of learning a perceptual skill: Nature 365:250-252.

Kellman P, Spelke E (1983) Perception of partly occluded objects in infancy. Cogn Psychol 15:483-524.

Kiorpes L, Movshon JA. (1996a) Amblyopia. A developmental disorder of the central visual pathways. Cold Spring Harbour Symposia on Quantitative Biology. Vol LXI. 39-48.

Kiorpes L, Walton PJ, O'Keefe LP, Movshon JA, Lisberger SG (1996b) Effects of early-onset artificial strabismus on pursuit eye movements and on neuronal responses in area MT of macaque monkeys. J Neurosci 16:6537-6553.

Kiorpes L (1992a) Development of vernier acuity and grating acuity in normally reared monkeys. Vis Neurosci 9:243-251.

Kiorpes L (1992b) Effect of strabismus on the development of vernier acuity and grating acuity in monkeys. Vis Neurosci 9:253-259.

Kohler W (1967) Gestalt psychology. Psychol Forsch. 31:18-30.

Konig P, Engel AK, Lowel S, Singer W (1993) Squint affects synchronization of oscillatory responses in cat visual cortex. Eur J Neurosci 5:501-508.

Kovács I, Kozma P, Fehér A, Benedek Gy (1999) Late maturation of visual spatial integration in humans. Proc Natl Acad Sci USA 96:12204-12209

Kovács I, Polat U, Norcia AM (1996a) Breakdown of binding mechanisms in amblyopia [ARVO Abstract]. Invest Ophthalmol Vis Sci 37:S130. Abstract nr 670.

Kovács I (1996b) Gestalten of today: early processing of visual contours and surfaces. Behav Brain Res 82:1-11.

Kovács I, Julesz B (1994) Perceptual sensitivity maps within globally defined visual shapes. Nature 370:644-646.

Kovács I, Julesz B (1993) A closed curve is much more than an incomplete one: Effect of closure in figure-ground segmentation. Proc Natl Acad Sci USA 90:7495-7497.

Kozma P, Deak A, Janaky M, Benedek G. (2001) Effect of late surgery for acquired esotropia on visual evoked potential. J Pediatr Ophthalmol Strabismus. 38:83-8.

Kozma P, Kovács I, Benedek Gy (1997) Late maturation (> 5 years) of long-range spatial interactions in humans. Perception Suppl ECVP 1997 Abstracts nr116a.

Kraft SP. Outcome criteria in strabismus surgery (1998) Can J Ophthalmol 33:237-239.

Kushner BJ (1998) Amblyopia. In Nelson LB, ed., Harley's Pediatric Ophthalmology, 4th ed., W.B. Saunders Co., USA, 125-139.

Kushner BJ (1994) Binocular field expansion in adults after surgery for esotropia. Arch Ophthalmol 112:639-643.

Kushner BJ (1988) Functional amblyopia: A purely practical pediatric patching protocol. Ophthalmol Ann 173-198.

Lakowski R, Aspinall PA (1969) Static perimetry in young children. Vision Res 9:305-311.

Lam GC, Repka MX, Guyton DL (1993) Timing of amblyopia therapy relative to strabismus surgery. Ophthalmology 100:1751-1756.

Leguire LE, Rogers GL, Bremer DL (1995) Flash visual evoked response binocular summation in normal subjects and in patients with early-onset esotropia before and after surgery. Doc Ophthalmol 89:277-286.

Leguire LE, Rogers GL, Bremer DL, Walson P, Hadjiconstantinou-Neff M (1992) Levodopa and childhood amblyopia. J Pediatr Ophthalmol Strabismus 29:290-298.

Leguire LE, Rogers GL, Bremer DL (1991) Visual evoked response binocular summation in normal and strabismic infants. Invest Ophthalmol Vis Sci 32:126-133.

Leguire LE, Rogers GL, Bremer DL (1990) Amblyopia: The Normal Eye is Not Normal. J Pediatr Ophthalmol Strabismus 27:32-38.

Lemire RJ, Loeser JD, Leech RW, Alvord EC (1975) Normal and Abnormal Development of the Human Nervous System. Harper and Row, Hagerstown, MD, 47.

LeVay S, Wiesel TN, Hubel DH (1980) The development of ocular dominance columns in normal and visually deprived monkeys. J Comp Neurol 191:1-51.

Levi DM, Carkeet A (1993) Amblyopia: A consequence of abnormal visual development. In Simons K, ed., Early Visual Development, Normal and Abnormal. Oxford University Press, New York, 391-408.

Lewis TL, Maurer D (1992) The development of the temporal and nasal visual fields during infancy. Vision Res 32:903-911.

Lewis TL, Maurer D, Brent HP (1989) Optokinetic nystagmus in normal and visually deprived children: Implications for cortical development. Can J Psychol 43:121-140.

Livingstone.M, Hubel D (1988) Segregation of form, color, movement, and depth: anatomy, physiology, and perception. Science 240:740-749.

Magoon EH, Robb RM (1981) Developmnent of myelin in human optic nerve and tract. Arch Ophthalmol 99:655-659.

Marg E, Freeman DN, Peltzman P, Goldstein PJ (1976) Visual acuity development in human infants: evoked potential measurements. Invest Ophthalomol Vis Sci 15:150-153.

Marotta JJ, De Souza JFX, Haffenden AM, Goodale MA (1998) Does a monocularly presented size-contrast illusion influence grip aperture? Neuropsychologia 36:491-497.

Matsuo H, Endo N, Yokoi T, Tomonaga M (1974) Visual field of the children. Ann Therap Clin Ophthalmol 25:186.

Mayer DL, Fulton AB, Cummings MF (1988) Visual field of infants assessed with a new perimetric technique. Invest Ophthalmol Vis Sci 29:452-459.

Mayer DL, Dobson V (1982) Visual acuity development in infants and young children, as assessed by operant preferential looking. Vision Res 22:1141-1151.

McGinnis JM (1930) Eye movements and optic nystagmus in early infancy. Genet Psychology Monogr 8:321-430.

Mein J, Harcourt B (1986) Diagnosis and Management of Ocular Motility Disorders. Blackwell, St. Louis, 181-193.

Milner AD, Goodale MA (1995) The Visual Brain in Action. Oxford University Press, Oxford.

Mitchell DE, Timney B (1984) Postnatal development of function in the mammalian visual system. In Darian-Smith I, ed., Handbook of Physiology The Nervous System III., American Physiological Society, Bethesda, MD, 507-555.

Mitchison GJ, Crick F (1982) Long axons within the striate cortex: their distribution, orientation, and patterns of connection. Proc Natl Acad Sci USA 79:3661-3665.

Morris RJ, Scott WE, Dickey CF (1993) Fusion after surgical alignment of longstanding strabismus in adults. Ophthalmology 100:135-138.

Moskowitz A, Sokol S (1983) Developmental changes in the visual system as reflected by the latency of the pattern reversal VEP. Electroencephalogr Clin Neurophysiol 56:1-15.

Nagele JR, Held R (1982) The postnatal development of monocular optokinetic nystagmus in infants. Vision Res 22:341-346.

Nelson CA, Horowitz FD (1987) Visual motion perception in infancy: A review and synthesis. In Salapatek P, Cohen L, eds., Handbook of Infant Perception. Vol. 2. Academic, New York, 123-153.

Nelson JI, Frost BJ (1985) Intracortical facilitation among co-oriented, coaxially aligned simple cells in the cat striate cortex. J Exp Brain Res 61:54-61.

Neville HJ, Bavelier D (2000) Specificity and plasticity in neurocognitive development in humans. In Gazzaniga MS, ed., The New Cognitive Neuroscience. 2nd ed., MIT Press, Cambridge, MA, 73-81.

Nuzzi G, Franchi A (1983) Binocular interaction in visual-evoked responses: summation, facilitation and inhibition in a clinical study of binocular vision. Ophthalmic Res 15:261-264.

O'Keefe M, Abdulla N, Bowell R, Lanigan B (1996) Binocular function and amblyopia after early surgery in infantile esotropia. Acta Ophthalmol Scand 74:461-462.

Ozanics V, Jakobiec FA (1985) Prenatal development of the eye and its adnexa. In Duane TD, Jager EA, eds., Biomedical Foundations of Ophthalmology. Harper and Row, Philadelphia.

Pennefather PM, Chandna A, Kovács I, Polat U, Norcia AM (1999) Contour detection threshold: repeatability and learning with "contour cards". Spatial Vision 12:257-266.

Petrig B, Julesz B, Kropfl W, Baumgartner G, Anliker M (1981) Development of stereopsis and cortical binocularity in human infants: electrophysiological evidence. Science 213:1402-1405.

Pettet MN, McKee SP, Gryzwacz NM (1998) Constraints on long-range interactions mediating contour detection. Vision Res 38:865-879.

Pettigrew JD (1982) Pharmacologic control of cortical plasticity. Retina 2:360-372.

Phelps G (1976) Amblyopia: Theories on its etiology. Ophthalmic Semin 1:1-19.

Poggio T, Fahle M, Edelman S (1992) Fast perceptual learning in visual hyperacuity. Science 256:1018-1021.

Polat U, Sagi D (1994) Spatial interactions in human vision from near to far via experience dependent cascades of connections. Proc Natl Acad Sci USA 91:1206-1209.

Reinecke RD (1978) Vision, amblyopia, and strabismus. In Feman SS, Reinecke RD, eds., Handbook of Pediatric Ophthalmology. Grune Stratton, New York, 1-11.

Rentschler I, Hilz R (1979) Abnormal orientation sensitivity in both eyes of strabismic amblyopes. Exp. Brain Res 37:187-191.

Rockland KS, Lund JS (1982) Widespread periodic intrinsic connections in the tree shrew visual cortex. Science 214:1532-1534.

Roelfsema PR, Konig P, Engel AK, Sireteanu R, Singer W (1994) Reduced synchronization in the visual cortex of cats with strabismic amblyopia. Eur J Neurosci 6:1645-1655.

Rogers GL, Chazan S, Fellows R, Tsou BH (1982) Strabismus surgery and its effect upon infant development in congenital esotropia. Ophthalmology 89:479-483.

Romano PE, Romano JA, Puklin JE (1975) Stereoacuity development in children with normal binocular single vision. Am J Ophthalmol 79:966-971.

Roy MS, Barsoum-Homsy M, Orquin J, Benoit J (1995) Maturation of binocular pattern visual evoked potentials in normal full-term and pretem infants from 1 to 6 months of age. Pediatr Res 37:140-144.

Sanke RF (1988) Amblyopia. Am Fam Phys 37:275-278.

Sawatari A, Callaway EM (1996) Convergence of magno- and parvocellular pathways in layer 4B of macaque primary visual cortex. Nature 380:442-446.

Schiffman HR (1996) Sensation and Perception. An Integrated Approach. 4th ed., Wiley, New York, 138-195.

Sclar G, Ohzawa I, Freeman RD (1986) Binocular summation in normal, monocularly deprived, and strabismic cats: visual evoked potentials. Exp Brain Res 62:1-10.

Sekuler R, Blake R (1994) Perception. 3rd ed., McGraw-Hill, New York, 141-180.

Shankle WR, Landing BH, Rafii MS, Schiano A, Chen JM, Hara J (1998a) Evidence for a postnatal doubling of neuron number in the developing human cerebral cortex between 15 months and 6 years. J Theor Biol 191:115-140.

Shankle WR, Romney AK, Landing BH, Hara J (1998b) Developmental patterns in the cytoarchitecture of the human cerebral cortex from birth to 6 years examined by correspondence analysis. Proc Natl Acad Sci USA 95:4023-4028.

Shapley R (1990) Visual sensitivity and parellel retinocortical channels. Annu Rev Psychol 41:635-658.

Shawkat FS, Kriss A, Timms C, Taylor DSI (1998) Comparison of pattern-onset, -reversal and -offset VEPs in treated amblyopia. Eye 12:863-869.

Shawkat FS, Kriss A (1997) Interocular interaction assessed by VEPs to pattern-onset, -reversal, and -offset in normally sighted and amblyopic subjects. Electroencephalogr Clin Neurophysiol 104:74-81.

Shea SL, Aslin RN, McCulloch D (1987) Binocular VEP summation in infants and adults with abnormal binocular histories. Invest Ophthalmol Vis Sci 28:356-365.

Simmers AJ, Gray LS (1999) Improvement of visual function in adult amblyope. Optom Vis Sci 76:82-87.

Simon JW, Calhoun JH (1998) Amblyopia. In Simon JW, Calhoun JH, eds., A Child's Eyes. A Guide to Pedatric Primary Care 95-100.

Sireteanu R (1996a) Development of the visual field: results from human and animal studies. In Vital-Durand F, Atkinson J, Braddick OJ, eds., Infant vision. 1st ed., Oxford University Press (EBBS Publication Series), New York, 17-32.

Sireteanu R, Rettenbach R (1996b) Texture discrimination and visual search: development, learning and plasticity. Klin Monatsbl Augenheilkd 208:3-10.

Sireteanu R, Rettenbach R (1995) Perceptual learning in visual search: fast, enduring, but non-specific. Vision Res 35:2037-2043.

Sireteanu R, Rieth C (1993) Texture segregation in infants and children. Behav Brain Res 49:133-139.

Sireteanu R, Kellerer R, Boergen KP (1984) The development of peripheral visual acuity in human infants. A preliminary study. Hum Neurobiol 3:81-85.

Sireteanu R (1982) Human amblyopia: Consequence of chronic interocular suppression. Hum Neurobiol 1:31-33.

Sireteanu R, Fronius M (1981) Naso-temporal asymmetries in human amblyopia: Consequence of long-term interocular suppression. Vision Res 21:1055-1063.

Sloper JJ, Collins AD (1998) Reduction in binocular enhancement of the visual evoked potentials during development accompanies increasing stereoacuity. J Pediatr Ophthalmol Strabismus 35:154-158.

Sokol S (1983) Abnormal evoked potential latencies in amblyopia. Br J Ophthalmol 67:310-314.

Sokol S (1980) Pattern visual evoked potentials: their use in pediatric ophthalmology. In Sokol S, ed., Electrophysiology and Psychophysics: Their Use in Ophthalmic Diagnosis. Little Brown, Boston, 251-268.

Sokol S, Dobson V (1976) Pattern reversal visual evoked potentials in infants. Invest Ophthalmol Vis Sci 15:58-62.

Sowell ER, Thompson PM, Holmes CJ, Jernigan TL, Toga AW (1999) In vivo evidence for post-adolescent brain maturation frontal and striatal regions. Nature Neurosci 2:859-861.

Starger DR, Birch EE (1986) Preferential-looking acuity and stereopsis in infantile esotropia. J Pediatr Ophthalmol Strabismus 23:160-165.

Stoner GB, Albright TD (1993) Image segmentation cues in motion processing: Implications for modularity in vision. J Cogn Neurosci 5:129-149.

Swan KC, Wilkins JH (1984) Extraocular muscle surgery in early infancy - anatomical factors. J Pediatr Ophthalmol Strabismus 21:44-49.

Tassinari G, Campara D, Balercia G, Chilosi M, Martignoni G, Marzi CA (1994) Magno- and parvocellular pathways are segregated in the

human optic tract. Neuroreport 5:1425-1428.
Taylor MJ, McCulloch DL (1992) Visual evoked potentials in infants and children. J Clin Neurophysiol 9:357-372.
Teller DY (2000) Visual development: Psychophysics, neural substrates, and causal stories. In Gazzaniga MS, ed., The New Cognitive Neuroscience. 2nd ed., MIT Press, Cambridge, MA, 73-81.
Teller DY, Bornstein MH (1987) Infant color vision and color perception. In Salapatek S, Cohen LB, eds., Handbook of Perception. Academic Press, New York, 185-236.
Teller DY (1982) Scotopic vision, color vision, and stereopsis in infants. Curr Eye Res 2:199-210.
Ts'o DY, Gilbert CD (1988) The organization of chromatic and spatial interactions in the primate striate cortex. J Neurosci 8:1712-1727.
Ungerleider LG, Mishkin M (1982) Two cortical visual systems. In Ingle DJ, Goodale MA, Mansfield RJW, eds., Analysis of Visual Behavior. MIT Press, Cambridge, MA, 549-586.
Uysal S, Renda Y, Topcu M, Erdem G, Karacan R (1993) Evoked potentials in full-term and premature infants: a comparative study. Childs Nerv Syst 9:88-92.
Vaegan, Taylor D (1979) Critical period for deprivation amblyopia in children. Trans Ophthalmol Soc UK 99:432-439.
von Noorden GK (1996) Binocular Vision and Ocular Motility. 5th ed., Mosby, USA, 216-254.
von Noorden GK (1985) Binocular Vision and Ocular Motility. Theory and Management of Strabismus. 3rd ed., USA, 240.
von Noorden GK (1961) Reaction time in normal and amblyopic eyes. Arch Ophthalmol 66:695.
Wali N, Leguire LE, Rogers GL, Bremer DL (1991) CSF interocular interactions in childhood amblyopia. Optom Vis Sci 68:81-87.
Wiesel TN (1982) Postnatal development of the visual cortex and influence of environment. Nature 299:583-591.
Wiesel TN, Hubel DH (1965) Comparison of the effects of unilateral and bilateral eye closure on cortical responses in kittens. J Neurophysiol 28:1029-1040.
Wiesel TN, Hubel DH (1963a). Effects of visual deprivation on morphology and physiology of cells in the cat's lateral geniculate body. J Neurophysiol 26:978-993.
Wiesel TN, Hubel DH (1963b) Single cell responses in striate cortex of kittens deprived of vision in one eye. J Neurophysiol 26:1003-1017.
Wilcox LM Jr., Sokol S (1980) Changes in the binocular fixation patterns and the visually evoked potential in the treatment of esotropia with amblyopia. Ophthalmology 87:1273-1281.
Willshaw HE, Keenan J (1991) Strabismus surgery in children: the prospects for binocular single vision. Eye 5:338-343.
Wilson HR, Wilkinson F (1998) Detection of global structure in Glass patterns: implications for form vision. Vision Res 38:2933-2947.
Wilson M, Quinn G, Dobson V, Breton M (1991) Normative values for visual fields in 4- to 12-year-old children using kinetic perimetry. J Pediatr Ophthalmol Strabismus 28:151-154.
Wortham EV, Greenwald MJ (1989) Expanded binocular peripheral visual fields following surgery for esotropia. J Pediatr Ophthalmol Strabismus 26:109-112.
Wright KW (1996) Surgical alignment in infants for congenital esotropia. Ophthalmology 103:700-701.
Wright KW (1995) Visual development, amblyopia, and sensory adaptations. In Wright KW, ed., Pediatric Ophthalmology and Strabismus, 1st ed., Mosby, USA, 45-57, 119-138.
Yakovlev PI, Lecours A (1967) The myelogeneticycles of regional maturation of the brain. In Minkowski A, ed., Regional Development of the Brain in Early Life. Blackwell, Oxford.
Yuodelis C, Hendrickson A (1986) A qualitative and quantitative analysis of the human fovea during development. Vision Res 26:847-855.
Zhang Z, Li S, Wu DZ (1993) Development of PVEP in infants and children. Yen Ko Hsueh Pao 9:23-30.

Volume 45(1-4):43-49, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

A comparative study of the conformational stabilities of trypsin and α-chymotrypsin

Mária L. Simon*, Kinga László, Márta Kotormán, Béla Szajáni

Department of Biochemistry, University of Szeged, Szeged, Hungary

ABSTRACT A comparative study was performed on the conformational stabilities of trypsin and α-chymotrypsin. At 45°C, trypsin was most stable at pH 3, while the highest stability of α-chymotrypsin was observed at pH 5. With both ester and amide substrates, trypsin displayed activation at pH 3. In the case of α-chymotrypsin, activation was detected at pH 5 only with the amide substrate. The time curves of heat inactivation were complex. For both enzymes, autolysis proceeded with the highest velocity at pH 8. The results obtained on α-chymotrypsin suggested consecutive reactions: the first step, heat denaturation of the protein, is followed by digestion of the damaged molecules. **Acta Biol Szeged 45(1-4):43-49 (2001)**

KEY WORDS

trypsin
α-chymotrypsin
conformational stability
autolysis
pH effect

Trypsin and α-chymotrypsin are well-known serine proteases (Desnuelle 1971; Keil 1971; Cohen et al. 1981; Journak and McPherson 1987). The serine proteases exhibit structural and chemical similarities, but their specificities are different (Polgár 1989).

According to early observations, trypsin is stable at pH 3 at low temperatures for weeks. It can be reversibly heat denaturated (Lazdunski and Delaage 1965). Lazdunski and Delaage (1967) investigated the effect of pH on the temperature-induced reversible denaturation of bovine trypsin. D'Albis (1970) conducted a thermodynamic study on the reversible thermodenaturation of trypsin in the pH range 1.0-3.4. The conformation of trypsin is well ordered between pH 7 and 8, but is considerably less ordered at more acidic or alkaline pH values. Both enzymes are susceptible to autolysis. Chymotrypsin A is most stable at pH 3, but even at this pH autolysis proceeds, although very slowly. At pHs lower than 3 or higher than 10, the enzyme undergoes conformational changes (Walsh and Wilcox 1970).

The conversion of trypsin to chymotrypsin and vice versa by site-directed mutagenesis is a model for protein engineers (Gráf et al. 1987; Heldstrom et al. 1992). Site-directed mutagenesis could modify the conformational stabilities of the enzyme derivatives. For an appraisal of the stability changes induced, a kinetic re-evaluation of the conformational stabilities of the parent enzymes appeared reasonable.

Heat denaturation experiments comprise a simple and inexpensive method of investigation of the conformational stabilities of proteins, and are useful for comparative studies, too. The present paper reports results on the heat inactivation of trypsin and α-chymotrypsin.

Accepted November 28, 2000
*Corresponding author. E-mail: simon@biocom.bio.u-szeged.hu

Materials and Methods

Materials

Bovine pancreas trypsin (EC 3.4.21.4), α-chymotrypsin (EC 3.4.21.1), N-benzoyl-L-arginine ethyl ester (BAEE), N-acetyl-L-tyrosine ethyl ester (ATEE), N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-carbobenzoxy-L-phenylalanine-p-nitroanilide (CPPNA) were purchased from Sigma-Aldrich Company (Budapest, Hungary). The specific activities were 40-60 units/mg for α-chymotrypsin and 10,000 units/mg for trypsin. All other chemicals were reagent grade products (Reanal, Budapest, Hungary).

Assays of enzyme activities

The activity of trypsin was measured by following the increase in absorbance at 253 nm (Geiger and Fritz 1984) in a reaction mixture (3 ml) containing 46.7 mM Tris/HCl buffer (pH 8.0), 19 mM $CaCl_2$ and 0.9 mM BAEE, the reaction being initiated by the addition of 5 units of enzyme. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 μM of BAEE per min at pH 8.0 and at 25°C. The activity measurements with BAPNA were carried out as follows: the reaction mixture contained 150 mM triethanolamine/HCl (pH 8.0), 15 mM $CaCl_2$, 0.8 mM BAPNA and 5-10 units of trypsin (Erlanger et al. 1961). The amount of p-nitroanilide released was monitored via the increase in absorbance at 410 nm. For the measurement of α-chymotrypsin activity, ATEE was used and the changes in absorbance at 237 nm were followed in a reaction mixture (3 ml) containing 40 mM Tris/HCl (pH 8.0), 50 mM $CaCl_2$

and 0.5 mM ATEE (Schwert and Takenaka 1955). The reaction was initiated by the addition of 4.5 units of enzyme. One unit of enzyme activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 μM of ATEE per min at pH 8.0 and at 25°C. For the activity determination with CPPNA as substrate, the reaction mixture (3.0 ml) contained 50 mM Tris/HCl (pH 8.0), 0.1 mM CPPNA in DMF and 5 units of α-chymotrypsin (Delmar et al. 1979). The amount of p-nitroanilide released was monitored via the increase in absorbance at 410 nm.

Stability tests

The pH dependencies of the conformational stabilities of trypsin and α-chymotrypsin were studied in the pH range 3-7 by using 0.1 M glycine/HCl buffer (pH 3), 0.1 M acetic acid/NaOH buffer (pH 4-5), 0.1 M citric acid/NaOH buffer (pH 6) and 0.1 M triethanolamine/HCl buffer (pH 7), respectively. Enzyme solutions of 0.1 and 1.0 mg/ml were prepared with the different buffers and incubated for 5 h at various temperatures. Aliquots of 100-200 μl were withdrawn and the residual activities were determined.

Measurements of ninhydrin-positive species

The appearance of ninhidrin-positive substances during heat treatment was followed quantitatively according to the procedure of Moore and Stein (1948).

Results

Effects of pH on stabilities of trypsin and a-chymotrypsin

The pH dependences of the stabilities of trypsin and α-chymotrypsin were studied at 45°C, both ester and amide substrates being used for the determination of residual activities. The protein concentration of the enzyme solution was 1 mg/ml.

For trypsin, similar results were obtained with either BAEE or BAPNA as substrate (Fig. 1), but the loss in amidase activity was somewhat faster, especially in the acidic media. At pH 3, activation was observed with both substrates (18-20% and 16-17%, respectively). Trypsin exhibited the highest stability at this pH. The inactivation was faster in the solutions with pH > 6 than that in the media with lower pHs.

The results obtained with α-chymotrypsin are depicted in Fig. 2. Significant differences were found in the stabilities of esterase (ATEE substrate) and amidase (CPPNA substrate) activities, especially at pH < 6. The highest stability of α-chymotrypsin was observed at pH 5. At this pH, the esterase activity was preserved for at least 4 h, while with the amide substrate activation of at most 24% was measured. Above pH 6 the inactivation was more rapid than that in the media with lower pHs. At pH 9, the enzyme was practically inactivated during the first 20 min of incubation.

Effects of temperature on stabilities of trypsin and α-chymotrypsin

The temperature dependence of the stability of α-chymotrypsin was studied at pH 4 in citrate buffer and at pH 7 in phosphate buffer, with ATEE as substrate. The protein concentration was 1 mg/ml. The results are presented in Fig. 3. At pH 4, the enzyme retained about 20% of its starting activity after incubation for 5 h at 50°C, while at pH 7 the enzyme practically lost all of its activity. At 55°C, the inactivation was complete during the first 20 min of incubation. In a 1 mg/ml solution, in phosphate buffer (pH 8) at 55°C, trypsin lost more than 90% of its initial esterase and amidase activities during a 5-min incubation.

Effects of protein concentration on stabilities of trypsin and α-chymotrypsin

The effects of 0.1 and 1 mg/ml protein concentrations on the stability of trypsin were studied in Tris/HCl buffer (pH 8) at 55°C, with both ester (BAEE) and amide (BAPNA) as substrates. After incubation for 2.5 min in the 0.1 mg/ml solution, trypsin had lost 55.6% of its original esterase activity, while in the 1 mg/ml solution only 11.5% of the starting activity was preserved. As regards the amidase activity, after incubation for 5 min in 0.1 mg/ml solution the activity loss was 61.6%, while in 1 mg/ml solution it was 93.2%. In the case of α-chymotrypsin, the effects of the protein concentration on the stability were studied at 50°C at pH 4 (sodium citrate) and pH 7 (potassium phosphate) in 0.1 and 1 mg/ml solutions, with ester (ATEE) and amide (CPPNA) as substrates. The inactivation in the 0.1 mg/ml solution was faster for both types of substrates (Fig. 4).

Autolysis of trypsin and α-chymotrypsin

Samples from the heat inactivation experiments were submitted to the ninhydrin test. The time curves of liberation of ninhydrin-positive species from trypsin are shown in Fig. 5. In 1 mg/ml solutions at pH 3 and 4, ninhydrin-positive substances could not be demonstrated, but at pH > 5 the autolysis proceeded rapidly. The highest rate was experienced at pH 8 in Tris/HCl buffer. The process involved at least two phases, a fast and a slower one. At that pH in the 0.1 mg/ml solution, the liberation of ninhydrin-positive substances was not detected at 45°C and 55°C.

In 0.1 mg/ml α-chymotrypsin solutions, heat-treated at 45°C and 50°C and at pH 4 and 7, respectively, ninhydrin-positive species were not liberated. In 1 mg/ml solutions at 45°C and in the pH range 3-4.5, ninhydrin-positive substances could likewise not be detected. At pH 6, a lag period was followed by the accelerated formation of autolysis products. At pH > 7, the time curves did not exhibit any lag period and the process proceeded rapidly. The maximum velocity was measured at pH 8 in potassium phosphate or Tris/HCl buffer

(Fig. 6). The autolysis involved at least two phases, similarly as for trypsin. At higher temperature (50°C), the lag period was observed only at pH 4 in the first 25 min of incubation (Fig. 7).

Discussion

Trypsin and α-chymotrypsin display close structural similarities. The backbone structure of the two proteases are highly homologous and the homology also extends to the catalytic triad and substrate-binding pocket regions (Steitz et al. 1969; Birktoft and Blow 1972; Polgár 1989). In spite of the structural similarities, however, there are significant differences in their conformational stabilities. In earlier work (Simon et al. 1998), we established that, in miscible polar solvents such as acetonitrile, ethanol and 1,4-dioxane, α-chymotrypsin has quite different behaviour from that of trypsin. The differences in the conformational stability are confirmed by the heat treatment experiments. At 45°C, trypsin is most stable at pH 3, while the highest stability of α-chymotrypsin was observed at pH 5. With both ester and amide substrates, trypsin shows activation at pH 3. In the case of α-chymotrypsin, activation was detected at pH 5 only with the amide substrate. The time curves of heat inactivation are complex for both enzymes, in consequence of the existence of different

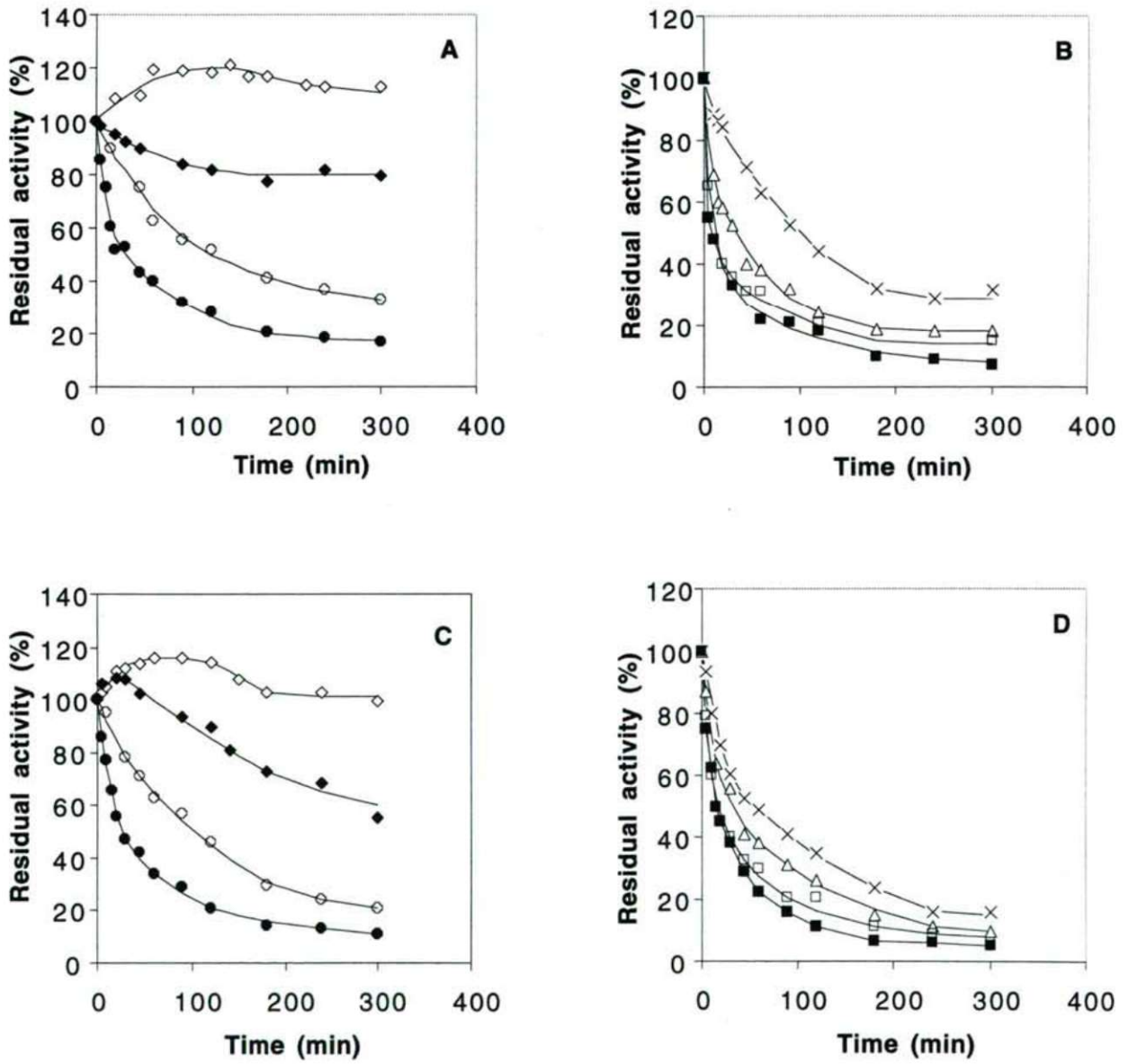


Figure 1. Effects of pH on inactivation of trypsin at 45°C. Protein concentration: 1 mg/ml. Substrates: BAEE (A, B) and BAPNA (C, D). Buffers (0.1 M): citrate (◊) pH 3, (♦) pH 4, (O) pH 5, (●) pH 6, phosphate (x) pH 6, (Δ) pH 7, (□) pH 8; borate (■) pH 9. For details, see text.

molecular forms. The stabilities of the different molecular forms of trypsin are temperature- and pH-dependent (Lazdunski and Delaage 1967). At 20°C, the acidification of trypsin from pH 8 to pH 0.5 results in the appearance of 3 reversible equilibria. The most important structural change in the alkaline range involves the unmasking of the abnormal tyrosines. This process is reversible, but is followed by an irreversible denaturation. α-chymotrypsin can exist in two major conformational states, only one of which is active. Stoesz and Lumry (1978) examined the pH and ionic strength dependence of the transition between the active and inactive forms. At low pH (pH 2.0-6.0), the equilibrium is very dependent on the salt concentration; high salt concentrations effectively stabilize the active conformation. This apparent stabilization is an artifact due to the dimerization of the active form of α-chymotrypsin. At pH 6.0-8.0, the dimerization does not occur. At pH > 6, the pH dependence can be described by a two-ionization mechanism at all ionic strengths. The self-association of α-chymotrypsin was studied by Pandit and Rao (1974). We suspect that the transient activa-

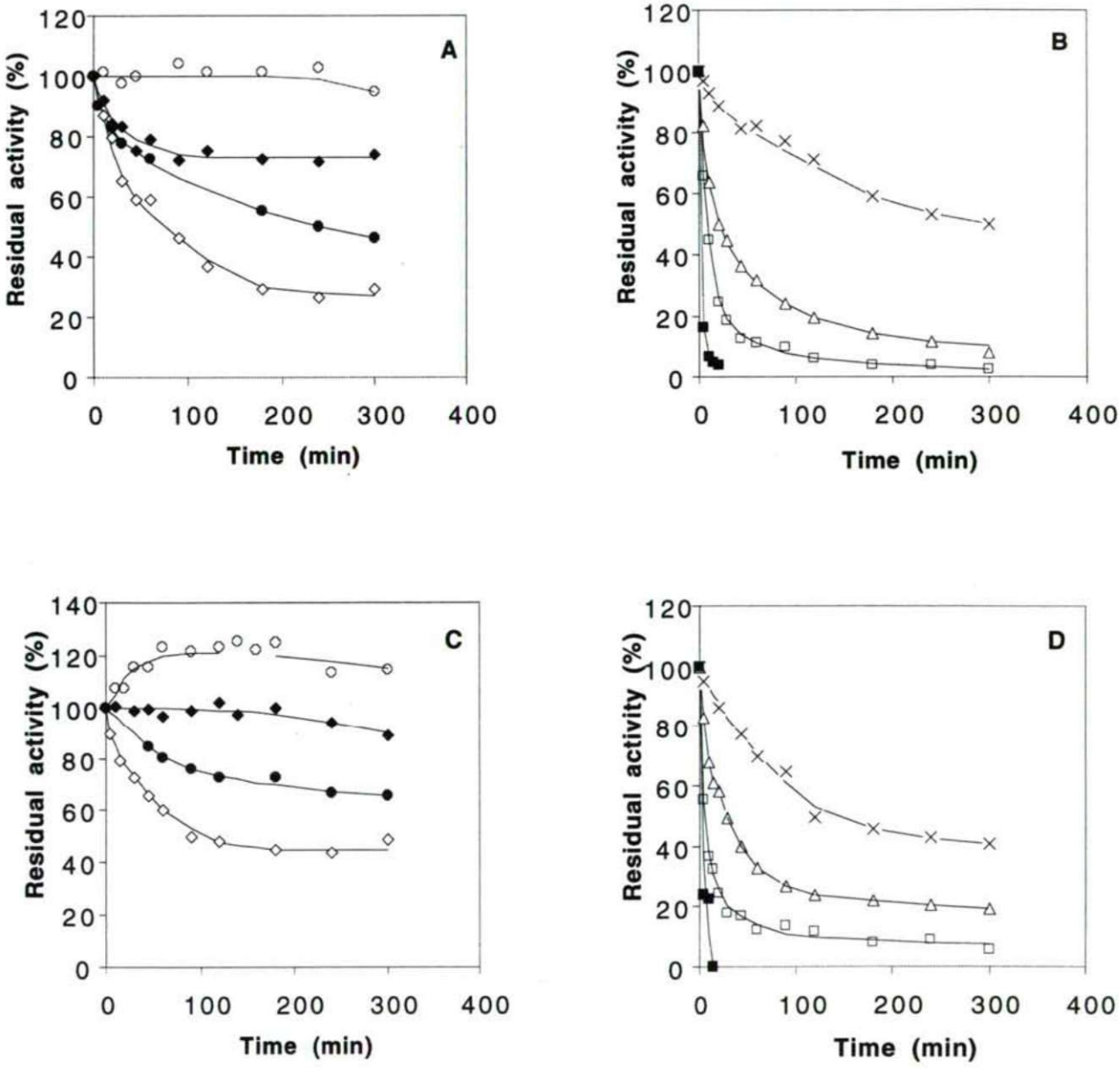


Figure 2. Effects of pH on inactivation of α-chymotrypsin at 45°C. Protein concentration: 1 mg/ml. Substrates: ATEE (A,B) and CPPNA (C, D). Buffers (0.1 M): citrate (◊) pH 3, (♦) pH 4, (O) pH 5, (●) pH 6, phosphate (x) pH 6, (Δ) pH 7, (□) pH 8; borate (■) pH 9. For details, see text.

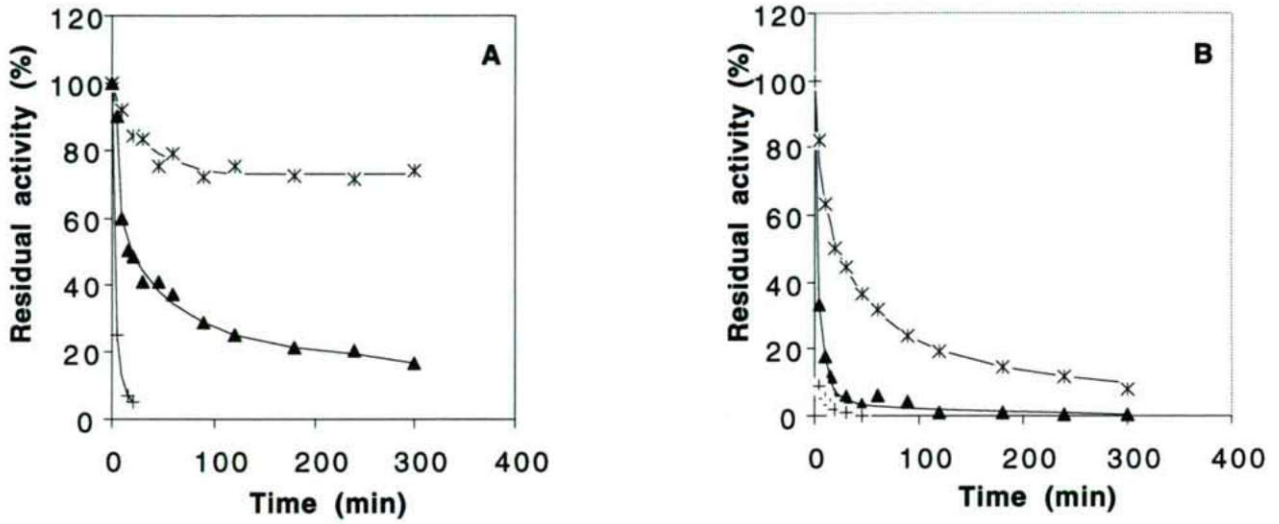


Figure 3. Effects of temperature on inactivation of α-chymotrypsin at pH 4 in citrate buffer (A) and at pH 7 in phosphate buffer (B) with ATEE as substrate. Protein concentration: 1 mg/ml. Temperatures: (*) 45°C, (▲) 50°C, (+) 55°C. For details, see text.

tions during the heat treatment stem from the rise of a molecular subform with a higher catalytic activity, but a lower stability.

The autolysis proceeds with the highest velocity at pH 8 for both enzymes. At pH 3 and 4, the liberation of the ninhydrin-positive substances from α-chymotrypsin molecules cannot be detected. A similar phenomenon was observed in 0.1 mg/ml solutions (in spite of the fast heat denaturation) at pH 4 and 7 at 45°C and 50°C for α-chymotrypsin and at pH 8 at 45°C and 50°C for trypsin. The detailed investigation by Kumar and Hein (1970) suggested that the mechanism of autolysis of α-chymotrypsin can be explained by an apparent second-order inactivation process. Autodigestion is chemically distinguishable from the process of denaturation. Our experimental results support these findings.

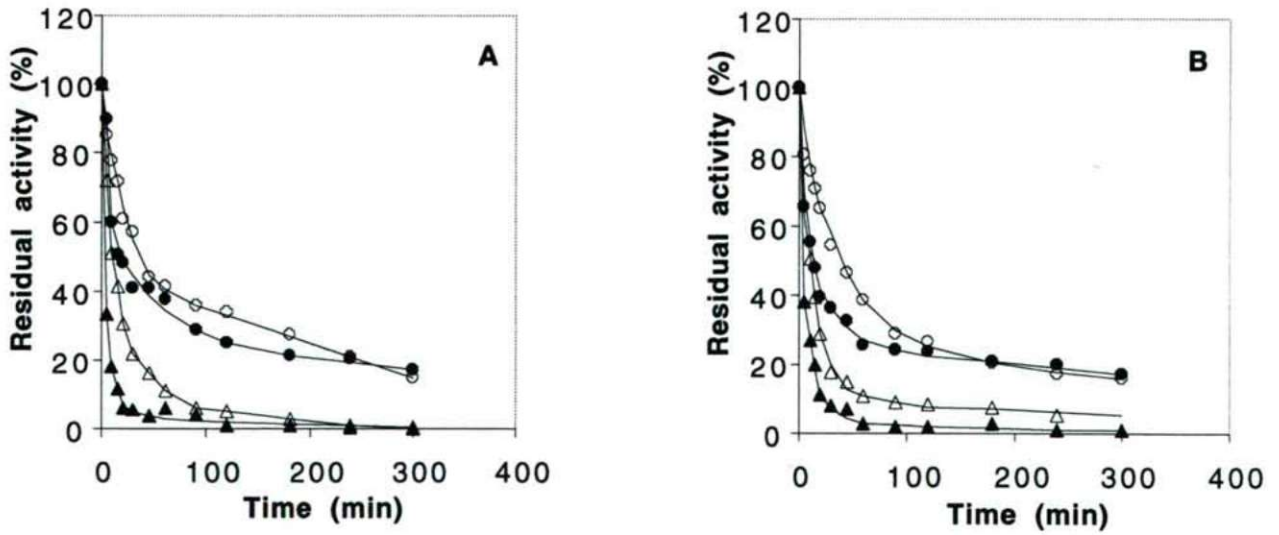


Figure 4. Effects of protein concentration on inactivation of α-chymotrypsin at 50°C at pH 4 in citrate buffer and at pH 7 in phosphate buffer, with ATEE (A) and CPPNA (B) as substrates. Protein concentrations and pHs: (O) 0.1 mg/ml and pH 4, (●) 1 mg/ml and pH 4, (Δ) 0.1 mg/ml and pH 7, (▲) 1 mg/ml and pH 7. For details, see text.

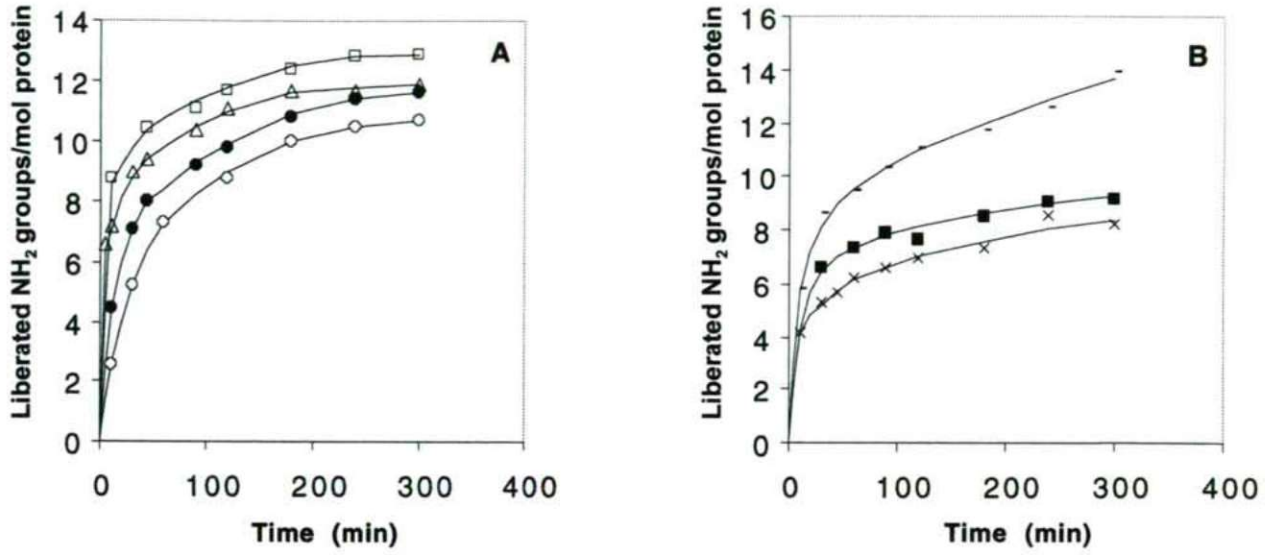


Figure 5. Effects of pH on autolysis of trypsin at 45°C. Protein concentration: 1 mg/ml. Buffers (0.1 M): citrate (O) pH 5, (●) pH 6; phosphate (Δ) pH 7, (□) pH 8; Tris/HCl (–) pH 8; borate (■) pH 9, (x) pH 10. For details, see text.

The results obtained on the heat denaturation of α-chymotrypsin at pH 6 and at 45°C point to consecutive reactions: the first step, heat denaturation, is followed by the digestion of the damaged molecules. Similar kinetics could not be observed for trypsin. We presume a higher sensitivity of trypsin for autodigestion, resulting in a very short, undetectable lag period.

References

Birktoft JJ, Blow DM (1972) Structure of chrystalline α-chymotrypsin. J Mol Biol 68:187- 240.

Cohen GH, Silverton EW, Davie DR (1981) Refined chrystal structure of γ-chymotrypsin in 1.9 A resolution. J Mol Biol 148:449-479.

D'Albis A (1970) Étude thermodinamique de la denaturation termique réversible de la trypsine entre pH 1.0 et 3.4. Biochim Biophys Acta 200:34-39.

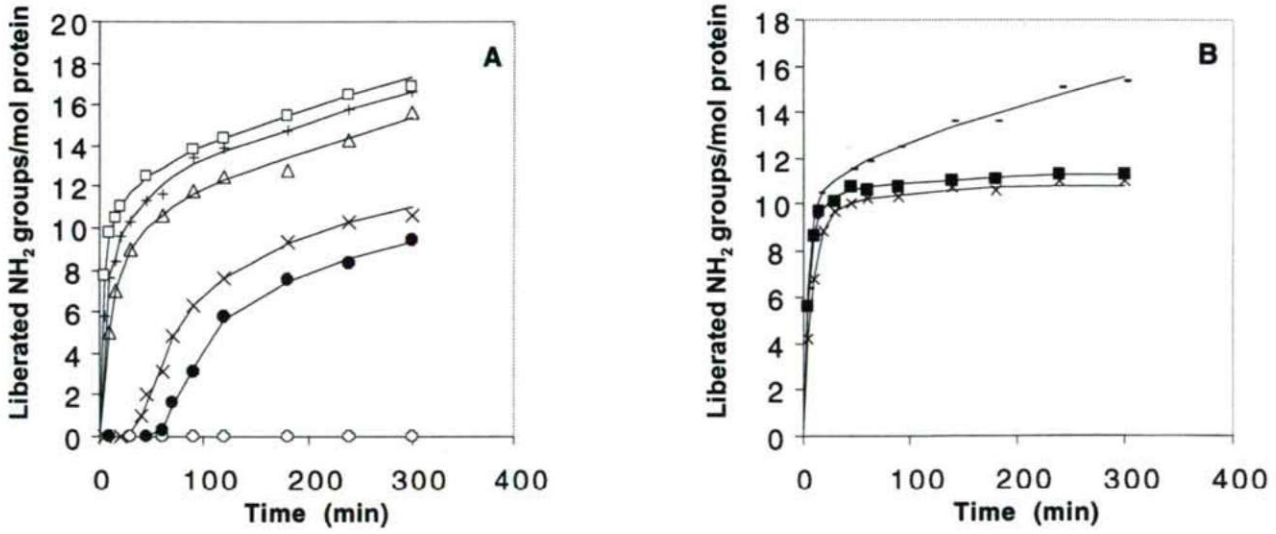


Figure 6. Effects of pH on autolysis of α-chymotrypsin at 45°C. Protein concentration: 1 mg/ml. Buffers (0.1 M): citrate (O) pH 5, (●) pH 6; phosphate (X) pH 6, (Δ) pH 7, (+) pH 7.5, (□) pH 8; Tris/HCl (–) pH 8; borate (■) pH 9, (x) pH 10. For details, see text.

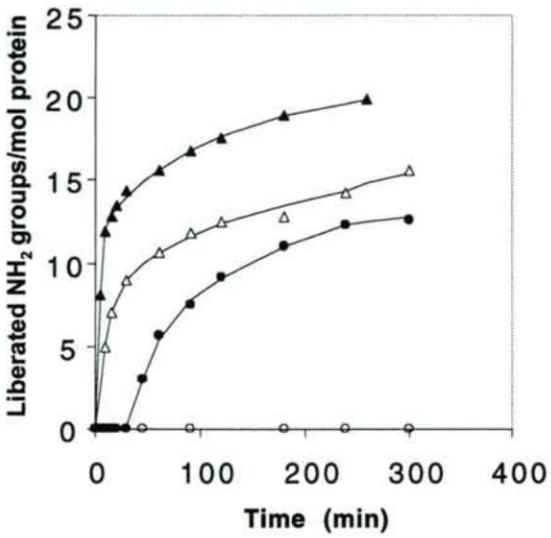


Figure 7. Effects of temperature on autolysis of α-chymotrypsin Enzyme concentration: 1 mg/ml. Buffers (0.1 M) and temperatures: citrate (O) pH 4 and 4 °C, (●) pH 45 and 50°C, phosphate (Δ) pH 7 and 45°C, (▲) pH 7 and 50 °C. For details, see text.

Delmar EG, Largman C, Brodrick JW, Geokas MC (1979) A sensitive new substrate for chymotrypsin. Anal Biochem 99:316-320.

Desnuelle P (1971) The structure of chymotrypsin. In Boyer PD, ed., The Enzymes, Academic Press, New York, 8:185-193.

Erlanger BF, Kokowsky M, Cohen W (1961) The preparation and properties of two new chromogenic substrates for trypsin. Arch Biochem Biophys 95:271-278.

Geiger R, Fritz H (1984) Trypsin, In Bergmeyer HU, ed., Methods of Enzymatic Analysis, Verlag Chemie, Weinheim, 5:119-123.

Gráf L, Craik ChS, Patthy A, Rozniak S, Fletterick RJ, Rutter WJ (1987) Selective alteration of substrate specificity by replacement aspartic acid-189 with lysine in the binding pocket of trypsin. Biochemistry 26:2616-2623.

Heldstrom L, Szilágyi L, Rutter WJ (1992) Converting trypsin to chymotrypsin: the role of surface loops. Science 225:1249-1253.

Jurnak FA, McPherson A, eds., (1987) Catalytic properties of trypsin, Biological macromolecules and assemblies: active site of enzymes, Wiley, New York, 377-385.

Keil B (1971) Trypsin. In Boyer PD, ed., The Enzymes, Academic Press, New York, 8:248- 275.

Kumar S, Hein GE (1970) Concerning the mechanism of autolysis of α-chymotrypsin. Biochemistry 9:291-297.

Lazdunski M, Delaage M (1965) Sur la morphologie des trypsines de porc et de bceuf étude des denaturations reversibles. Biochim Biophys Acta 105:541-561.

Lazdunski M, Delaage M (1967) Étude structurale du trypsinogéne et de la trypsine. Les diagrammes d' état. Biochim Biophys Acta 140:417-434.

Moor S, Stein WH (1948) Photometric ninhydrin method for use in the chromatography of amino acids. J Biol Chem 176:367-388.

Polgár L (1989) Mechanism of protease action. CRC Press, Boca Raton

Schwert GW, Takenaka Y (1955) A spectrophotometric determination of trypsin and chymotrypsin. Biochim Biophys Acta 16:570-575.

Simon LM, László K, Vértesi A, Bagi K, Szajáni B (1998) Stability of hydrolytic enzymes in water-organic solvent systems. J Mol Catal B: Enz 4:41-45.

Steitz TA, Henderson R.D, Blow M (1969) Structure of christalline α-chymotrypsin. J Mol Biol 46:337-348.

Stoesz JD, Lumry RW (1978) Refolding transition of α-chymotrypsin: pH and salt dependence. Biochemistry 17:3693-3699.

Pandit MW, Narasinga Rao MS, (1974) Studies on self-association of proteins. The self- association of α-chymotrypsin at pH 8.3 and ionic strength 0.05. Biochemistry 13:1048- 1053.

Walsh KA, Wilcox PE (1970) Serine proteases, In Perlmann GE, Lorand L, eds., Methods in Enzymology, Academic Press, New York, 19:31-42.

Volume 45(1-4):51-55, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Unmasking of latent synaptic connections in the cortex of the rat, elicited by facial nerve transection

Tamás Farkas*, József Toldi

Department of Comparative Physiology, University of Szeged, Szeged, Hungary

ABSTRACT Peripheral nerve injury elicits plastic changes in the cortex, resulting in reorganization of the somatotopic representation maps. These processes begin within minutes after nerve injury, and last for weeks. Although the mechanisms leading to these plastic changes are not known in a detail, a number of results suggest that the key element in the starting of these processes is a decrease in the function of the cortical GABAergic system, which allows the unmasking of pre-existing but normally silent synapses. The somatosensory and motor cortices of the rat brain are involved in strong and mutual interaction. This study of the early changes induced in this relationship by unilateral facial nerve transection (N7x) revealed that the disinhibition of associational and commissural connections caused by N7x allowed the appearance and enhancement of potentials in the motor cortices on both sides, evoked by right-side trigeminal stimulation, though these responses were strictly lateralized in normal animals. In response to the juxtacellular application of GABA and muscimol, reversal was observed in a small population of neurons (3 out of 84) tested with microelectrode recordings and pressure microinjections. These results suggest that a peripheral nerve injury (N7x) rapidly induces $GABA_A$ receptor-dependent disinhibition in the cortex.
Acta Biol Szeged 45(1-4):51-55 (2001)

KEY WORDS

facial nerve
nerve injury
cortical disinhibition
unmasking of latent synapses
pressure microinjection

Peripheral nerve injury (both afferents and efferents) causes time-predictable changes in the representation borders of the related cortical areas (Calford et al. 1996; Sanes and Donoghue 2000). However, little is known concerning the mechanisms inducing these changes. As regards the somatosensory cortex (SI), it is known that partial deafferentation is followed by a decrease in inhibition driven by the afferent inputs (Calford and Tweedale 1991). A decrease in GABA immunostaining in the cortex has also been reported (Alloway et al. 1989).

Similar changes have been observed in the motor cortex (MI) after de-efferentation. It is known that there is a reorganization of MI representations through associational connections within days and weeks after nerve injury (Donoghue et al. 1990; Sanes et al. 1990). It was recently established that these denervation-induced changes appear in two phases. The initial phase starts minutes after nerve injury, *e.g.* 4 minutes after unilateral transection of the facial nerve (N7x), intracortical microstimulation of the "de-efferented" MI area (vibrissa region) elicits vibrissal movements ipsilateral to the stimulation through the activation of pre-existing commissural connections (Toldi et al. 1996). Those branches of the facial nerve which are involved in vibrissal movements (and are cut) are known to be entirely efferent in rats (Semba and Egger 1986). During this short period of time, only the modification of pre-existing synaptic connections may occur, *e.g.* through the horizontal projections which traverse the representation borders (Huntley 1997). The mechanisms of this phenomenon remain to be elucidated. It is known that in the MI of the rat the somatosensory feedback of the forelimb participates in the maintenance of its representation borders (Sanes et al. 1992). Between the primary SI and MI, there is a strong functional interaction, described by both morphological and electrophysiological methods (Miyashita et al. 1994; Izraeli and Porter 1995; Farkas et al. 1999). These observations suggest a mutual interdependence between the somatosensory and motor systems of the rat. We considered it of interest to learn more about these modifying cortical connections after N7x.

We present evidence here that denervation-induced disinhibition allows the unmasking of pre-existing associational connections between the SI and MI in the contralateral hemisphere, and the unmasking of commissural connections between the MIs in both hemisphere. Evidence is additionally reported for a cellular mechanism possibly involved in disihibitional processes induced by peripheral nerve injury.

Accepted January 3, 2001
*Corresponding author. E-mail: tfarkas@bio.u-szeged.hu

Materials and Methods

Surgery

A total of 21 Sprague-Dawley rats (200-250 g) of either sex were involved in this study. The surgical procedures used followed the protocol for animal care approved by the Hungarian Health Committee (1998) and the international guidelines (European Communities Council Directives, 86/609/EEC). The animals were anaesthetized with an initial intraperitoneal dose of a Ketavet and Rompun mixture (10.0 mg/100 g, Cp-Pharma, and xylasine, 0.8 mg/100 g, Bayer AG, respectively). Maintenance doses were administered at hourly intervals. The right facial nerve (including its postauricular branch) was exposed and laid onto a special hook-shaped knife. The edges were retained, which allowed normal conduction. The nerve was transsected later, during the electrophysiological recordings. The MIs on both sides were exposed by craniotomy from about 2 mm to 5 mm anterior to the bregma, and from 0.5-5 mm lateral to the midline, according to the stereotaxic atlas of Paxinos and Watson (1982). The dura mater was removed and the cortical surface was covered with warm paraffin oil (Sigma, St. Louis, MO, USA) in order to prevent cooling and drying. The core temperature was maintained at 37°C and the animals were kept at rest for at least 30 min after surgery.

Stimulation and electrophysiological recordings

The right vibrissa pad was stimulated with a bipolar needle electrode at threshold intensity (1 Hz frequency, 300 ms duration, 10 ms delay and 200-250 μA amplitude). The stimulation elicited clear movements of several vibrissae and evoked potentials in the vibrissal field of the contralateral MI. At the beginning of each experiment, cortical mapping of the evoked potentials was performed to find the appropriate point with evoked potentials with the highest amplitude in the MI contralateral to the stimulation (the punctum maximum, usually localized 2 mm lateral and rostral to the bregma). Continuous parallel recordings of evoked potentials were carried out from the punctum maximum of the contralateral MI and from its homotopic ipsilateral point. In another series of experiments, extracellular unit recordings were performed at the punctum maximum of the contralateral MI. The glass microelectrodes were filled with 2.5 M NaCl (impedance 15-20 MW). Action potentials with amplitudes at least three times higher than the noise were recorded and analysed. In this series of experiments, N7x was performed during the surgery. In some experiments, pyramidal tract stimulation was carried out for identification of the cells being recorded (Landry et al. 1984). For this stimulation, the ventral surface of the pyramidal tract was exposed, and a small flat bipolar electrode was gently placed on the surface of the pyramidal

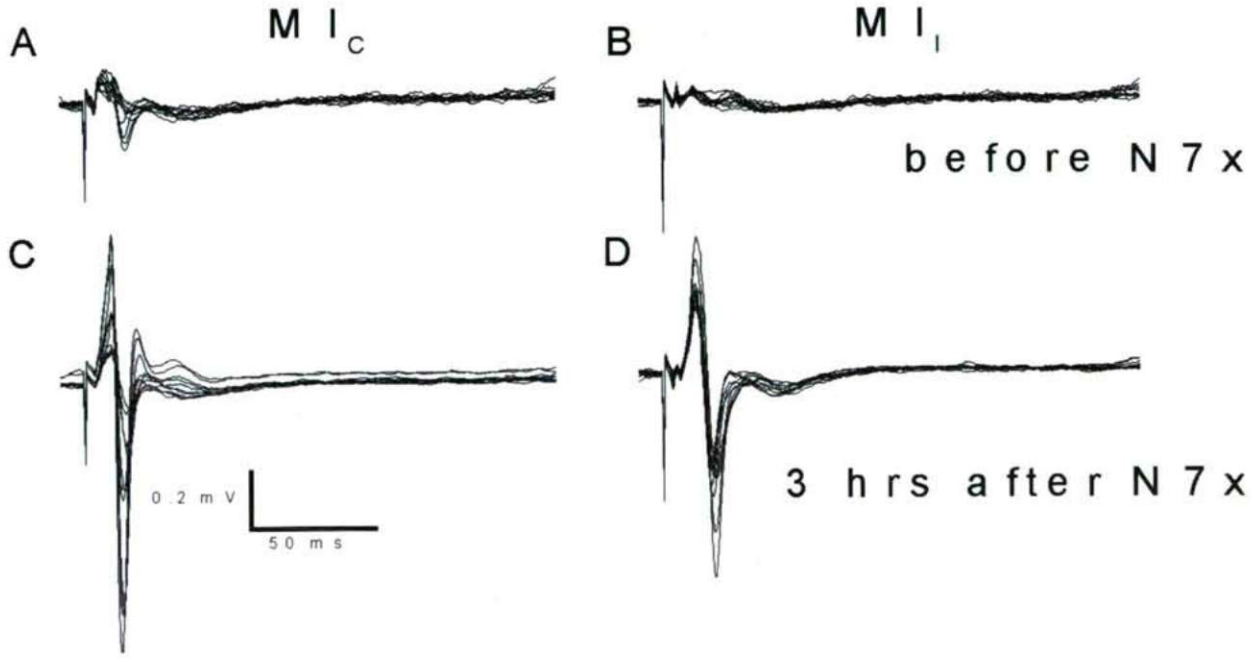


Figure 1. Evoked potentials of an N7x animal, recorded from the MIs on both sides: MI_C: primary motor cortex contralateral to both N7x and trigeminal stimulation. MI_I: primary motor cortex ipsilateral to both N7x and trigeminal stimulation. Each set of evoked potentials contains 10 averages cumulated from 60 sweeps. **A, B**: potentials recorded before N7x in MI_C and MI_I, respectively. **C, D**: potentials recorded 3 hours after N7x in MI_C and MI_I, respectively. Note that before N7x there were no evoked potentials in MI_I. Three hours after N7x, evoked potentials with large amplitude could be observed in the MIs in both hemispheres.

tract. Evoked potentials and unit responses were digitized and fed into a computer via an interface (Digidata 1200, pClamp604 software, Axon, Union City, CA, USA). Each registration contained 60 sweeps. Peristimulus time histograms (bin width 1.44 ms) were produced from unit responses.

Drug application

GABA or muscimol (10^{-3} M, dissolved in sterile saline) was applied juxtacellularly by pressure microinjection. For details of the electrode manufacturing, see Farkas et al. (1996). Briefly, two glass microelectrodes were glued together: one sharp electrode for recording and another one with a broken tip for drug application. Under a light microscope, the electrodes were adjusted so as to give a distance of ≤40 µm between their tips. At the optimal pressure, 25-40 pl of solution was applied.

Results

In the control animals, the electrical vibrissa pad stimulation elicited evoked potentials in the contralateral MI, but not in the ispilateral MI (Fig. 1 A and B). The parameters of the evoked potentials (shape, latency and amplitude) were identical to those published earlier (Toldi et al. 1999). Interestingly, the evoked responses in the contralateral MI were rapidly modified after N7x. A few hours after N7x, the amplitude of these potentials was highly enhanced and the latencies of all components had shortened (Fig. 1 C). More dramatic changes were observed in the ipsilateral MI. A few minutes after N7x, evoked potentials could be elicited in the MI ipsilateral to the stimulation, and their amplitude increased and remained high until the end of the experiments (Fig. 1 D).

A total of 84 neurons were isolated in the punctum maximum area of the vibrissal field in the contralateral MI after N7x. The response pattern and laminar distribution of the recorded neurons with responses to peripheral stimulation seemed to be identical to those described earlier in normal rats (Farkas et al. 1999). As expected, the application of GABA or muscimol inhibited most of these neurons (not shown here). However, a small percentage of the neurons (3 of the 84) displayed a reversal in response to the juxtacellular application of GABA or muscimol. Figure 2 shows an example. This pyramidal cell, recorded at a depth of 1,100 µm, responded to both pyramidal tract and peripheral vibrissa pad stimulation (Fig. 2 A, B). The application of GABA elevated the responses for 6 minutes (from 41 to 59 spikes/bin width, peak value taken from the first activation period: Fig. 2 C, D and E). The neuron did not display any signs of injury at any time during the registration period.

Discussion

Stimulation of the infraorbital nerve elicits well-characterized responses in the primary somatosensory (barrel) cortex of the rat (Welker 1976). The MI receives a somatotopically organized input from the SI (Miyashita et al. 1994; Izraeli and Porter 1995), and more than one-third of the MI units are also driven by peripheral somatosensory stimulation (Farkas et al. 1999). In normal rats, these evoked responses are strictly lateralized: they can be seen only in the MI contralateral to the stimulation, even during a prolonged period of stimulation (Farkas et al. 1999). After N7x, however, in 80% of the cases, the evoked responses were facilitated in the MI contralateral to the stimulation, and responses also appeared in the ipsilateral MI (Fig. 1). A few minutes after N7x, evoked responses could be observed and gradually increased in amplitude in the ipsilateral MI. In the rat, the MIs in both hemispheres are strongly interconnected by commissural fibres (Záborszky and Wolff 1982). The application of picrotoxin to the SI contralateral to the stimulation in normal rat elicited changes in the MIs in both hemispheres that were similar to the changes produced by N7x: the evoked potentials in the MIs on both sides were facilitated (Toldi et al. 1996, 1999), and some hours after picrotoxin application, large, type-II spike-waves (Coenen et al. 1995) could be detected. All these observations suggest that $GABA_A$ receptor-dependent changes take place in the SI and MI after N7x, which allow the unmasking of associative and commissural connections resulting in the appearance and facilitation of evoked potentials in the MIs in both hemispheres.

It is well known that GABA is the major inhibitory transmitter in the central nervous system of adult mammals (Macdonald and Olsen 1994). The opening of Cl^- channels in the cortical neurons of adult animals allows Cl^- to enter the cell because of the concentration gradient (inside 7 mM, outside 130 mM; Zigmond et al. 1999), which causes hyperpolarization of the cell membrane (Sivilotti and Nistri 1991). Under certain circumstances, however, GABA may act as an excitatory transmitter, *e.g.* after stimulation with high frequency, after axotomy, in heat stress or in a hypo-osmotic situation (van den Pol et al. 1996; Kaila et al. 1997; Taira et al. 1997). In these cases, an increased intracellular Cl^- concentration was found, which resulted in a Cl^- efflux (instead of an influx) during $GABA_A$ receptor activation (Ben-Ari et al. 1997; Kaila et al. 1997). In slice preparations containing a small ischaemic lesion, Neumann-Haefelin et al. (1995) showed that the neurons recorded closely to the infarct had a less negative membrane potential, and that there was a weaker GABA-mediated synaptic inhibition. From other studies, it is known that only a small decrease (10%) in GABAergic inhibition can lead to epileptic seizures through the horizontal spreading of cortical activation (Chagnac

Amitai and Connors 1989). Our own results demonstrate that in a few cases GABA or its agonist can serve as an excitatory transmitter (Fig. 2), possibly through a reversed Cl^- gradient. The presumed reversed Cl^-gradient of some neurons after N7x has been supported by a new histochemical technique. A transient Cl^- accumulation after N7x has been revealed in subpopulations of cortical neurons in the MIs and SIs in both hemispheres (Toldi et al. 2000). For technical reasons, at present we cannot prove that the Cl^--rich cells are identical with those of neurons which display a reversal in response to GABA, and we cannot prove that the Cl^- accumulation is the reason for the reversal effect of GABA. Disinhibition of a pyramidal cell (*e.g.* by inhibition of a GABA interneuron) cannot be excluded, which may also display a reversal in GABA effect in extracellular recording.

The clinical relevance of this type of studies is obvious. The injury of a peripheral nerve or an injury within the CNS (*e.g.* an ischaemic infarct) may cause serious malfunctions of the nervous system (phantom pain or synkinesia), which could be repaired at least partly during the recovery (Merzenich and Jenkins 1993; Heiss and Graf 1994; Johansson and Grabowski 1994; Nudo and Friel 1999). We hypothesize that the decrease in the GABAergic inhibition favours recovery by facilitating plastic changes, but in parallel with this the risk of seizures becomes greater.

Conclusions

After a facial nerve cut, there is a possibility for the appearance and enhancement of potentials in the MIs in both

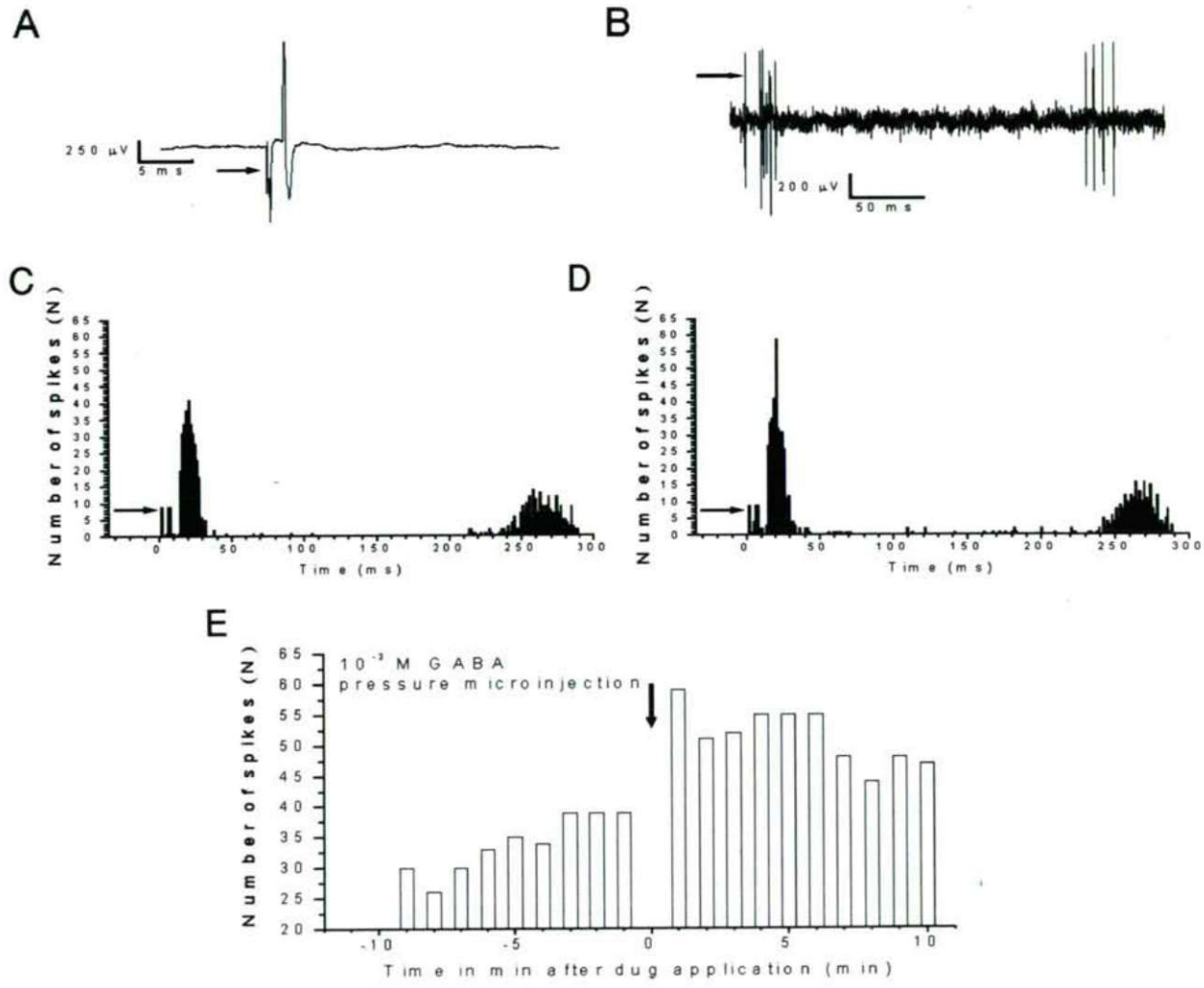


Figure 2. Responses of an identified pyramidal neuron to 10^{-3} M GABA pressure microinjection. **A**: The pyramidal tract stimulation elicited an antidrom response with short latency (2-3 ms). **B**: After identification of a pyramidal cell, its somatosensory input was tested with contralateral vibrissa pad stimulation. Poststimulus time histograms of a neuron show the evoked unit activity 1 min before **(C)** and after **(D)** drug application. The motor cortical neurons displayed the typical response pattern described previously (Farkas et al. 1999). **E**: The change in evoked unit activity elicited by GABA microinjection. This graph shows the time course of the change in spike activity of the neuron presented in **C** and **D**. Horizontal arrows point to the stimulation artefacts (in **A**, **B**, **C** and **D**), while the vertical arrow in **E** indicates the drug application.

hemispheres, evoked by unilateral trigeminal stimulation. The bases of the new activity pattern are the cortical associational and commissural connections, which are partly disinhibited after peripheral nerve injury. Our observations at a cellular level suggest that the reason for this phenomenon might be a $GABA_A$ receptor-dependent decrease in inhibition, caused by the transient Cl^- accumulation of some pyramidal cells in the de-efferented MI.

References

Alloway KD, Rosenthal P, Burton H (1989) Quantitative measurements of receptive field changes during antagonism of GABAergic transmission in primary somatosensory cortex of cats. Exp Brain Res 78:514-532.

Ben-Ari Y, Khazipov R, Leinekugel X, Caillard O, Gaiarsa JL (1997) GABAA, NMDA and AMPA receptors: a developmentally regulated 'menage a trois'. Trends Neurosci 20:523-529.

Calford MB, Clarey JC, Tweedale R (1996) Short-term plasticity in adult somatosensory cortex. In: Morley J, ed., Neural aspects of tactile sensations, Advances in Physiology Series. Elsevier Science Publishers B.V., 1-49.

Calford MB, Tweedale R (1991) C-fibres provide a source of masking inhibition to primary somatosensory cortex. Proc R Soc Lond B Biol Sci 243:269-275.

Chagnac Amitai Y, Connors BW (1989) Horizontal spread of synchronized activity in neocortex and its control by GABA-mediated inhibition. J Neurophysiol 61:747-758.

Coenen AML, Blezer EHM, van Luijtelaar ELJM (1995) Effects of the GABA-uptake inhibitor tiagabine on electroencephalogram, spike-wave discharges and behaviour of rats. Epilepsy Res 21:89-94.

Donoghue JP, Suner S, Sanes JN (1990) Dynamic organization of primary motor cortex output to target muscles in adult rats. II. Rapid reorganization following motor nerve lesions. Exp Brain Res 79:492-503.

Farkas T, Kis Z, Toldi J, Wolff JR (1999) Activation of the primary motor cortex by somatosensory stimulation in adult rats is mediated mainly by associational connections from the somatosensory cortex. Neurosci 90:353-361.

Farkas T, Korodi K, Toldi J (1996) Stimulus-dependent muscarinic effects on evoked unit activity in the rat barrel cortex. Neurosci Lett 212:61-64.

Heiss WD, Graf R (1994) The ischemic penumbra. Curr Opin Neurol 7:11-19.

Huntley GW (1997) Correlation between patterns of horizontal connectivity and the extent of short-term representational plasticity in rat motor cortex. Cereb Cortex 7:143-156.

Izraeli R, Porter LL (1995) Vibrissal motor cortex in the rat: connections with the barrel field. Exp Brain Res 104:41-54.

Johansson BB, Grabowski M (1994) Functional recovery after brain infarction: plasticity and neural transplantation. Brain Pathol 4:85-95.

Kaila K, Lamsa K, Smirnov S, Taira T, Voipio J (1997) Long-lasting GABA-mediated depolarization evoked by high-frequency stimulation in pyramidal neurons of rat hippocampal slice is attributable to a network-driven, bicarbonate-dependent K^+ transient. J Neurosci 17:7662-7672.

Landry P, Wilson CJ, Kitai ST (1984) Morphological and electrophysiological characteristics of pyramidal tract neurons in the rat. Exp Brain Res 57:177-190.

Macdonald RL, Olsen RW (1994) GABAA receptor channels. Annu Rev Neurosci 17:569-602.

Merzenich MM, Jenkins WM (1993) Reorganization of cortical representations of the hand following alterations of skin inputs induced by nerve injury, skin island transfers, and experience. J Hand Ther 6:89-104.

Miyashita E, Keller A, Asanuma H (1994) Input-output organization of the rat vibrissal motor cortex. Exp Brain Res 99:223-232.

Neumann-Haefelin T, Hagemann G, Witte OW (1995) Cellular correlates of neuronal hyperexcitability in the vicinity of photochemically induced cortical infarcts in rats in vitro. Neurosci Lett 193:101-104.

Nudo RJ, Friel KM (1999) Cortical plasticity after stroke: implications for rehabilitation. Rev Neurol (Paris) 155:713-717.

Paxinos G, Watson C (1982) The rat brain in sterotaxic coordinates. Academic Press, Sydney.

Sanes JN, Donoghue JP (2000) Plasticity and primary motor cortex. Annu Rev Neurosci 23:393-415.

Sanes JN, Suner S, Donoghue JP (1990) Dynamic organization of primary motor cortex output to target muscles in adult rats. I. Long-term patterns of reorganization following motor or mixed peripheral nerve lesions. Exp Brain Res 79:479-491.

Sanes JN, Wang J, Donoghue JP (1992) Immediate and delayed changes of rat motor cortical output representation with new forelimb configurations. Cereb Cortex 2:141-152.

Semba K, Egger MD (1986) The facial "motor" nerve of the rat: control of vibrissal movement and examination of motor and sensory components. J Comp Neurol 247:144-158.

Sivilotti L, Nistri A (1991) GABA receptor mechanisms in the central nervous system. Prog Neurobiol 36:35-92.

Taira T, Lamsa K, Kaila K (1997) Posttetanic excitation mediated by GABA(A) receptors in rat CA1 pyramidal neurons. J Neurophysiol 77:2213-2218.

Toldi J, Laskawi R, Landgrebe M, Wolff JR (1996) Biphasic reorganisation of somatotopy in the primary motor cortex follows facial nerve lesions in adult rats. Neurosci Lett 203:179-182.

Toldi J, Farkas T, Perge J, Wolff JR (1999) Facial nerve injury produces a latent somatosensory input through recruitment of the motor cortex in the rat. Neuroreport 10: 2143-2147.

Toldi J, Farkas T, Kis Z, Perge J, Grimm U, Wolff JR (2000) Modifying somatotopy in the primary motor cortex after facial nerve lesions in adult rats. J Physiol (London) 526:p.71.

van den Pol AN, Obrietan K, Chen G. 1996. Excitatory actions of GABA after neuronal trauma. J Neurosci 16:4283-4292.

Welker C (1976) Receptive fields of barrels in the somatosensory neocortex of the rat. J Comp Neurol 166:173-189.

Záborszky L, Wolff JR (1982) Distribution patterns and individual variations of callosal connections in the albino rat. Anat Embriol (Berl) 165:213-232.

Zigmond M, Bloom F, Landi S, Roberts J, Squire L (1999) Fundamental neuroscience. Academic Press, San Diego.

Volume 45(1-4):57-64, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

C-kit positive cellular network in normal human bowel and in motility disorders

László Németh[1,2]*, Péter Németh[1], Edit Oszlács[3], Prem Puri[2]

[1]Department of Paediatric Surgery, University of Szeged, Szeged, Hungary, [2]Children's Research Centre, Our Lady's Hospital for Sick Children, Dublin, Ireland, [3]Paediatric General Practical Service of Szeged, Szeged, Hungary

ABSTRACT C-kit positive interstitial cells of Cajal (ICC) appear to have a key role in the normal motility function and development of intestine. They are pacemaker cells, which facilitate active propagation of electrical events and neurotransmission in the bowel wall. The cellular network of ICC is connected by gap junctions to each other and to the smooth muscle cells. Nitrergic innervation is considered to be the most important part of nonadrenergic, noncholinergic innervation in the enteric nervous system. Gut innervation has a complex three-dimensional system that is difficult to appreciate in thin sections. Whole-mount preparation produces a three-dimensional picture to better demonstrate the structure of neuronal networks and their relationship of branching and interconnecting nerve fibres to each other and to the neighbouring tissues. Histochemical and immunohistochemical staining methods combined with whole-mount preparation technique provides a new aspect for studying bowel innervation and distribution of the crucial intracellular molecular architecture. The characteristic profiles of c-kit positive cellular network and nitrergic innervation and their relationship with the smooth muscle fibres in normal gut and in motility disorders provide a morphological basis for investigating intestinal motility disorders. **Acta Biol Szeged 45(1-4):57-64 (2001)**

KEY WORDS

c-kit
interstitial cell of Cajal
normal gut
motility disorders

Although it is now more than one hundred years since Cajal described small fusiform cells with prominent nuclei as forming network in the gastrointestinal tract, many questions about these cells remain unanswered (Rumessen 1993). In the human bowel the interstitial cells of Cajal (ICC) are localized at the level of myenteric plexus between the longitudinal and circular muscle layer, in the deep muscular plexus in the innermost part of the circular muscle layer, and within the circular muscle layer itself (Faussone-Pellegrini 1990; Wester 1999b). Morphological studies have been suggested three major functions for ICCs: (1) as pacemaker cells in the muscles of the gastrointestinal tract, (2) they facilitate active propagation of electrical events, and (3) they mediate neurotransmission (Sanders 1996). Recent reports indicate that transmembrane tyrosine-kinase receptor c-kit is essential for the development and function of the ICCs (Maeda 1992; Rumessen 1996). The immunoreactivity of c-kit is present in various cell types, but in the gut, c-kit is expressed only in ICCs and mast cells (Sanders 1996). ICCs appear to have a key role in the normal function and development of intestine (Hagger 1998; Toma 1999).

The normal motility of the gastrointestinal tract depends on the enteric nervous system (ENS), the muscle layers and the ICCs. It is currently accepted that the cellular network of ICCs is connected by gap junctions to each other and to the smooth muscle cells (Thuneberg 1989; Sanders 1996). Gap junctions are transmembrane channels that allow the exchange of ions, metabolites, and other small molecules (less than 1000 D) including second messengers such as cAMP, inositol triphosphate and Ca^{2+}, between the cytoplasms of adjacent cells (Garfield 1995; Simon 1999). Gap junctional intercellular communication is considered to play a crucial role in the maintenance of homeostasis and morphogenesis, cell differentiation, and growth control in multicellular organisms (Lau 1996; Morley 1997). The basic unit conforming the gap junction channels is a protein called connexin. Fourteen different homologous connexin proteins have been identified and characterized in rodents and eight in humans (Li 1993; Nakamura 1998; Nagaoka 1999). Connexin43 is regarded as the major gap junction protein in mammalians (Nakamura 1998; Mambetisaeva 1999).

The pathophysiology of Hirschsprung's disease (HD) is not fully understood. The most important histological finding in aganglionic colon is the absence of ganglion cells, which normally co-ordinate muscular activity by balancing the motor effects of the preganglionic cholinergic fibres and the inhibitory influence of the postganglionic adrenergic fibres

Accepted July 24, 2001
*Corresponding author. E-mail: nemethla@pedia.szote.u-szeged.hu

(Puri 1993, 1997, 1998). Several authors have examined distribution of c-kit positive cells in the normal and HD bowel and reported marked reduction in c-kit immunopositive cells in the aganglionic bowel (Vanderwinden 1996a; Yamataka 1997; Horisawa 1998).

The whole-mount preparation technique produces a three-dimensional picture to better demonstrate the structure of neuronal networks and their relationship of branching and interconnecting nerve fibres to each other and to the neighboring tissues (Wester 1999). This method is extremely useful for morphological analysis of nerve distribution in luminal organs such as the gastrointestinal tract in both normal condition and in motility disorders (Nemeth 2000b). The optical limitation of conventional light microscopy in the whole-mount preparation technique is that the image viewed is the sum of a sharp-in-focus region and structures outside the focal plane. In confocal laser scanning microscopy, only in-focus light is imaged through a pinhole, whereas out-of-focus light is rejected by the edge of the pinhole. Confocal microscopy thus provides non-invasive optical serial sections through thick biological samples with preserved three-dimensional structure (Delorme 1998).

The aim of this study was to examine the distribution of ICCs and their relationship to the enteric nervous system in normal human gut and in motility disorders.

Materials and Methods

Bowel specimens

Full thickness small and large bowel specimens were obtained at autopsy from 18 children who died of non-gastrointestinal diseases (age: 3 months-12 years). Bowel specimens were opened along the antimesenteric border and were rinsed in PBS and cut into 1x1-cm pieces containing all layers. Subsequently, they were fixed in diluted Zamboni solution (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer; pH7.3, 900 mOsM), stored overnight at 4°C, rinsed in phosphate buffer saline for 8 hours at 4°C and stored at -70°C in small plastic tubes until use.

Whole-mount preparation

Whole-mount preparation was made in each specimen using fine-pointed forceps, microsurgical scissors and dissection microscope. Initially, the mucosa-submucosa was removed in one layer followed by separation of muscular layers from the submucosal layer. Subsequently, the circular muscle layer was peeled off meticulously fibre by fibre from the longitudinal muscle layer to which the myenteric plexus is adherent. (Fig. 1). The separated layers were fixed without stretching with fine pointed pins on Sylgard silicone elastomer tray (Dow Corning Europe, La Hulpe, Belgium) with the myenteric plexus on the surface of the longitudinal muscle layer. Similar fixation was made on the separated submucosa layer.

Double staining with NADPH-diaphorase histochemistry and c-kit immunohistochemistry

For histochemical staining with NADPH diaphorase, the tissue specimens were incubated in 1 mg/mL β-NADPH (Sigma, Dorset, UK), 0.25 mg/mL nitroblue tetrazolium (Sigma), and 0.5 % Triton-X in 0.05 mol/L Tris-HCl buffer (pH 7.6) at 37°C until having a robust staining in the nitrergic neurons and nerves. The specimens were then rinsed 3 x 10 min in PBS solution. Thereafter the whole tissue specimens were incubated in mouse c-kit antibody (Novo Castra) in dilution rate 1:50 and 10% normal rabbit serum (DAKO) overnight at 4°C. After being rinsed in PBS twice the tissue samples were incubated with a biotinylated secondary rabbit anti mouse antibody (DAKO) in dilution 1:200 for 2h. The specimens were rinsed again and incubated in ABComplex/HRP (DAKO) for 60 min and developed in 3.3'-diaminobenzidine tetrahydrochloride (Sigma, London, UK) for about 6 min. Finally the specimens were rinsed and embedded in Glycergel mounting medium (DAKO), covered by glass and investigated with traditional light microscope.

Double immunofluorohistochemistry with nNOS and c-kit

After finishing the whole-mount preparation the specimens were rinsed in PBS solution. Before the standard steps of the immunofluorescein staining procedure the specimens were incubated in 0.5% Triton-X in 0.05 mol/L Tris-HCl buffer (pH 7.6) solution at 37°C for 4 h. Rinsing twice in PBS the tissues were incubated in nNOS (NOS1) antibody in dilution 1:50 (Santa Cruz, CA, USA) with 10% normal goat serum to prevent the aspecific linking overnight in 4°C. After being rinsed in PBS twice the tissue samples were incubated with Texas Red labeled goat anti rabbit antibody (Molecular

Figure 1. Typical appearance of a c-kit positive Interstital ICCs in the normal human bowel wall using confocal laser scanning microscopy. Original magnification: x 1000.

Probes, Leiden, The Netherlands) in dilution rate 1:50 as a secondary reagent for 2 h at room temperature (RT). Thereafter the whole tissue specimens were rinsed twice and incubated in mouse c-kit antibody (Novo Castra) in dilution 1:50 and 10% normal rabbit serum (DAKO) overnight at 4°C. As secondary reagent in this step FITC conjugated rabbit anti mouse immunoglobulin (DAKO) was used in dilution 1:20 for 2h at RT. The tissue specimens were embedded into fluorescence mounting medium (DAKO) and investigated with confocal laser scanning microscopy.

Confocal laser scanning microscopy

Specimens were observed using an upright laser scanning confocal microscope (BIO-RAD 2000, Hamil Hamsted, UK) with immersion objectives (x40 NA 0.45, NPL Fluotar or x63 NA 0.75). Tissue specimens were excited using Krypton/Argon laser with excitation and barrier filters set for individual fluorophores according to their specific excitation-emission spectra. (λ= 568 nm, 488 nm, 647 nm). The emitted light was detected by a photomultiplier tube and converted, via an analogue-to-digital converter (BIO-RAD), into a digital pixelated image (512 x 512 picture elements). The detection pinhole was set for use different objectives accordingly. Offset and gain settings were determined at the start of each experiment and kept constant throughout, with laser powered recorded each time.

Three-dimensional pictures were created by overlapping from 12 to 16 images obtained from a single optical section thus obtaining a reconstruction of the sample through its thickness. Serial optical sections were collected at 0.72 µm intervals from the affected area forming a 3-D XYZ image.

Results

Double staining with NADPH-diaphorase histochemistry and c-kit immunohistochemistry

The whole-mount preparation demonstrated the three-dimensional cellular network of c-kit positive cells in the muscle coat of the human bowel. Typical appearance of c-kit positive ICCs was a stellate-like cell with 3-4 long and several short processes protruding in every direction (Fig. 1). The three-dimensional network formed by the c-kit positive cells was mainly located between the longitudinal and circular muscle layers of the bowel wall and at the innermost part of the circular muscle layer (Fig. 2A). This network was very dense between the circular and longitudinal muscle layers. There was also a dense network of the c-kit positive cells at the innermost part of the circular muscle layer. The c-kit positive cells were connected to each other mainly by their long processes. The c-kit positive cells located between the circular muscle fibres had only two long processes running always parallel with the muscle fibres and from the long processes several short processes ran into the muscle fibres making close connection with them. There were no morphological differences observed in the c-kit positive cellular network between the different levels of gastrointestinal tract and in different age groups.

The whole-mount preparation facilitated three-dimensional visualization of the meshlike network of NADPH-diaphorase positive nerve fibres in the myenteric plexus. There was a mesh of nerve bundles with ganglia, containing clusters of ganglion cells between the two muscle layers of the bowel wall. Morphology of the NADPH-diaphorase

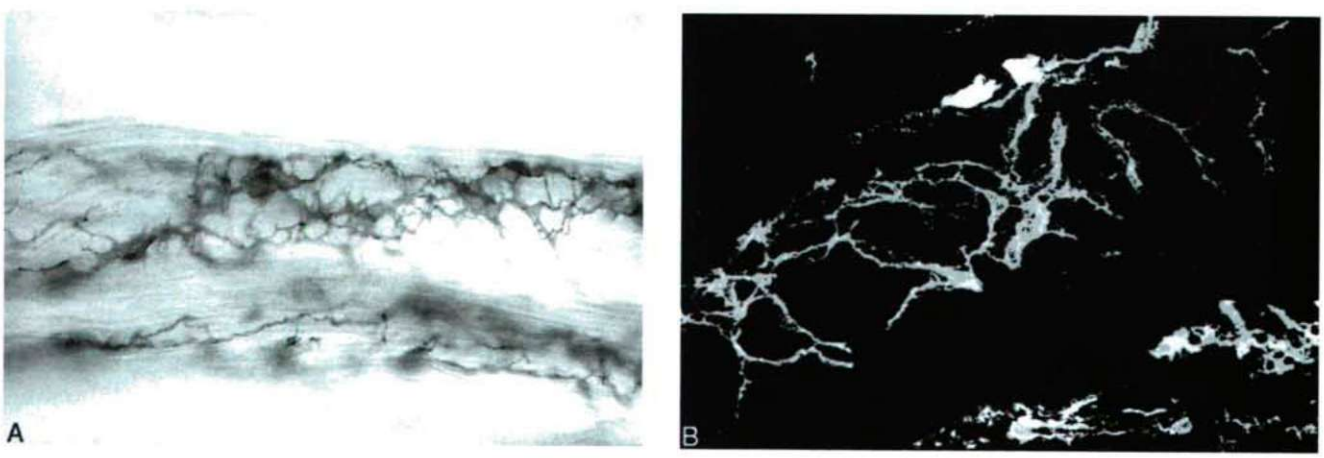


Figure 2. C-kit positive ICCs in the normal human bowel wall as forming a regular cellular network between the smooth muscle fibres **A.** Traditional ABC c-kit immunohistochemistry using light microscopy. Original magnification: x 430. **B.** c-kit immunofluorohistochemistry using confocal laser microscopy. Original magnification: x 430.

positive neuronal network varied between different parts of human gastrointestinal tract. The density of the typical architecture of the meshlike neuronal network increased in distal direction from the duodenum to the large bowel. The dense c-kit positive cellular network, located between longitudinal and circular muscle layer and at the innermost part of circular muscle layer intermingled with the myenteric plexus. Between the circular muscle fibres there was abundance of fine NADPH-diaphorase positive nerve fibres and c-kit positive cellular network running parallel with the muscle. The NADPH-diaphorase positive myenteric plexus was surrounded by a reticular network of c-kit positive ICCs (Fig. 3A).

Double immunofluorohistochemistry with nNOS and c-kit using confocal laser scanning microscopy

In whole-mount preparations immunopositivity to anti nNOS protein antibody was observed between the circular and longitudinal muscle layers as a three-dimensional network of neurons and nerve fibres. The expression of NADPH and the nNOS in enteric neurons and nerves were found in the same location in the entire human gastrointestinal tract. Immunoreactivity to anti c-kit protein antibody was observed in the space between the circular and longitudinal muscle layers surrounding the myenteric plexus (Fig. 3B). The c-kit positive cells formed a dense, three-dimensional network, connected to each other by the long processes (Fig. 2B). C-kit immunopositive cells were also observed between the smooth muscle fibres of the circular muscle layer. The long processes of c-kit positive ICCs ran parallel with the muscle fibres and from the long processes short fine processes made connections with the muscle fibres and c-kit positive cells.

Connexin43

Immunoreactivity to anti-connexin43 antibody was densely and homogeneously distributed as immunopositivity within the whole circular muscle layer. Strong connexin43 immunoreactivity was also seen at the innermost layer of the circular muscle and between the two muscle layers. On double staining the connexin43 expression was present in the cell body and processes of the c-kit positive ICCs (Fig. 4).

Aganglionic segment and transitional zone of Hirschsprung's disease bowel

c-kit

In the aganglionic segment of HD bowel, the c-kit positive ICC were sparse and localised mainly around the nerve trunks between the circular and longitudinal muscle layers (Fig. 5). In the transitional zone c-kit positive ICCs were higher in number than in aganglionic bowel but less than in normal bowel.

Connexin43

There was no expression of anti-Connnexin43 in the circular muscle layer and between the two muscle layers of the aganglionic bowel. In the transitional zone of the HD bowel specimens the immunolocalisation of connexin43 protein was in the processes of the c-kit positive ICCs but not in the cell bodies (Fig. 6).

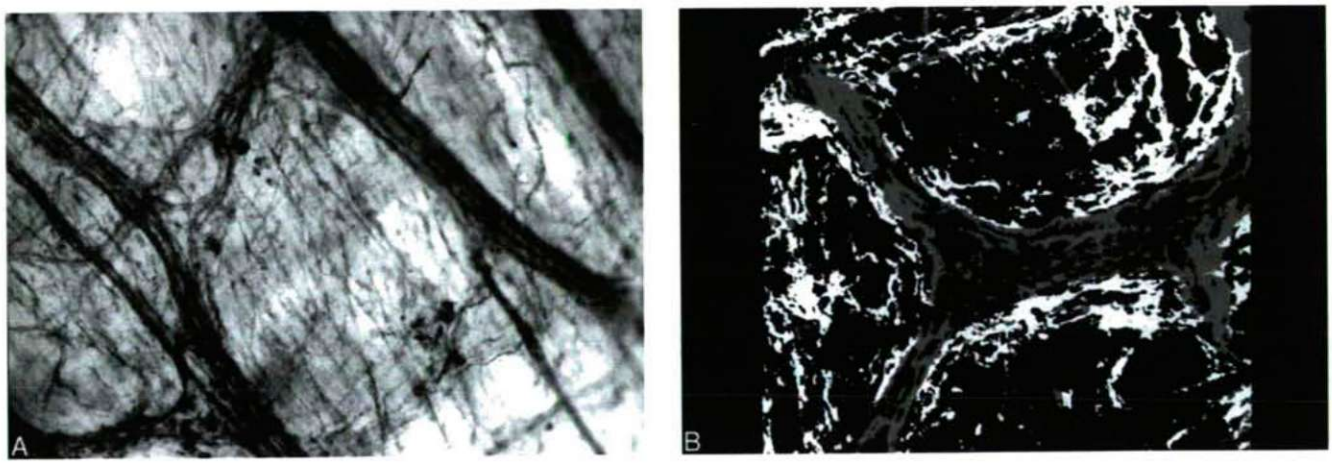


Figure 3. A. Whole-mount preparation of a normal human bowel wall using NADPH-diaphorase histochemistry and c-kit immunohistochemistry and conventional light microscopy. C-kit positive ICCs are forming a dense network surrounding the NADPH diaphorase positive myenteric plexus. Original magnification: x 200. **B.** Whole-mount preparation of a normal human bowel wall using double immunohistochemistry with nNOS and c-kit and confocal laser scanning microscopy. C-kit positive ICCs are forming a regular network around the nNOS positive nerve fibres of the myenteric plexus. Original magnification: x 200.

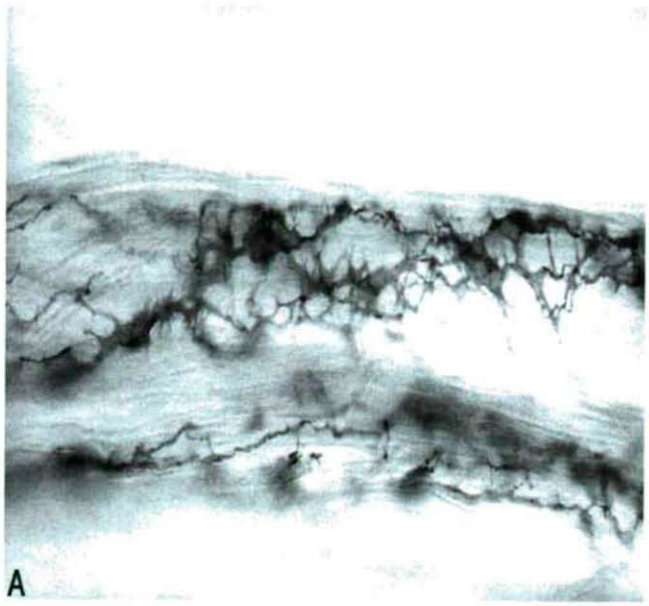


Figure 4. Expression of connexin43 in the ganglionic (normal) part of patient with Hirschsprung's disease. The immunolocalisation of connexin43 is present in the ICCs. Confocal laser scanning microscopy. Original magnification: x 300.

Discussion

Gut innervation has a complex three-dimensional structure that is difficult to appreciate in thin sections, which show only a part of the plexuses, neurons and glial cells. Whole-mount preparation is an elegant technique for the visualization of the myenteric plexus. It provides a method for the study of three-dimensional morphology of the meshwork of nerve fibres and neurons in detail (Fekete 1995; Wester 1999; Nemeth 2000b). Several investigators have used this technique in specimens from the human gastrointestinal tract with various staining methods ranging from silver impregnation to enzyme histochemistry and immunocytochemistry (Ferri 1982; Mebis 1990; Wedel 1998). The optical limitation of conventional light microscopy in the whole-mount preparation technique is that the image viewed is the sum of a sharp-in-focus region and structures outside the focal plane. In confocal laser scanning microscopy, only in-focus light is imaged through a pinhole, whereas out-of-focus light is rejected by the edge of the pinhole. Confocal microscopy thus provides non-invasive optical serial sections through thick biological samples with preserved three-dimensional structure (Delorme 1998).

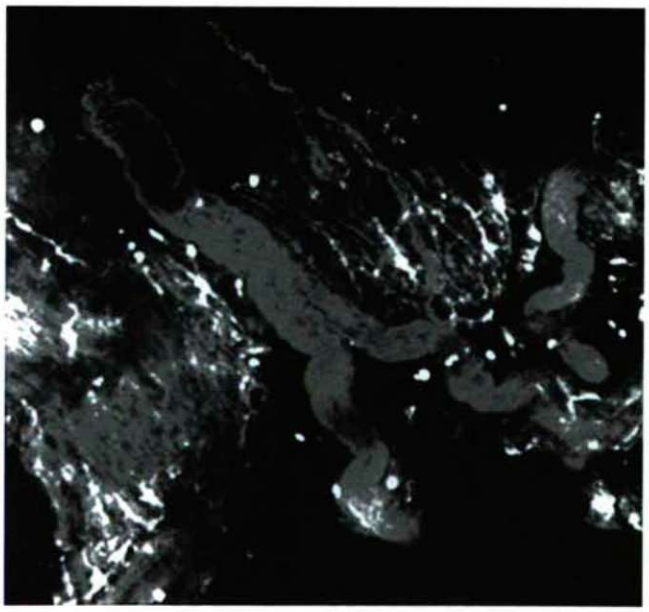


Figure 5. Whole-mount preparation of aganglionic large bowel segment from a patient having Hirschsprung's disease using double immunohistochemistry with nNOS and c-kit. ICCs are present in reduced number around the hypertrophied nerve trunks replacing the normal myenteric plexus Confocal laser microscopy. Original magnification: x 200.

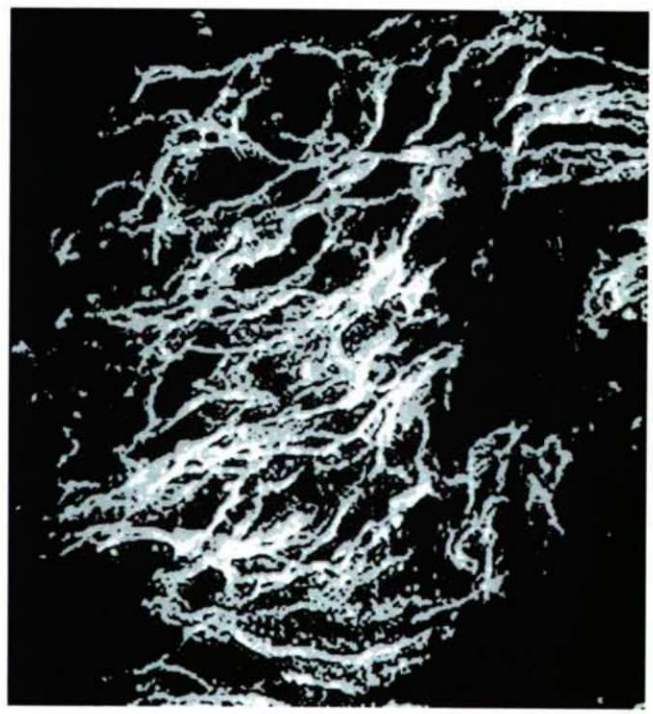


Figure 6. Expression of connexin43 in the transitional zone of large bowel segment from a patient with Hirschsprung's disease. The immunolocalisation of connexin43 is present only in the long processes of ICCs. Confocal laser scanning microscopy. Original magnification: x 430.

Nitric oxide, whose formation is catalyzed by NOS from L-arginine, has been recognized as an inhibitory neurotransmitter to mediate smooth muscle relaxation in the mammalian gastrointestinal tract (Desai 1991). In 1990, Bult et al. first provided evidence that NO is released on stimulation of enteric nonadrenergic noncholinergic (NANC) nerves. Furthermore, the effects of exogenously administered NO or its precursors (L-arginine or nitrosocystein) mimic both the electrophysiological and mechanical effects of NANC nerve stimulation in the intestines of different animal species. In addition, inhibition of NO synthesis by L-arginine analogues or inactivation of NO by oxyhemoglobin have been demonstrated to block NANC nerve-induced relaxation in numerous parts of gastrointestinal system.

The morphology and distribution of ICCs have previously been difficult to study, as standard staining procedures for conventional light microscopy do not disclose this cell type. The c-kit proto-oncogene encodes a transmembrane protein tyrosine kinase receptor and the antibody raised against c-kit protein opened a new chapter in the investigation of c-kit positive cells (Maeda 1992; Sanders 1996). It is well recognised that ICCs are required for the generation of the smooth muscle electrical slow wave (Torihashi 1995). The electrical slow wave determines smooth muscle contractile activity. In the absence of an electrical slow wave, contractile activity is decreased and irregular, resulting in decreased intestinal transit. ICCs are intercalated between nerve terminals and smooth muscle cells, providing a means of transducing signals from neurotransmitters and mediating neurotransmission (Xue 1994). Recently, it has been suggested that ICC may produce NO and amplify inhibitory neurotransmission. Absence or reduction in number of ICCs has been implicated in several disorders of human gastrointestinal motility, including hypertrophic pyloric stenosis, HD, intestinal pseudoobstruction and slow transit constipation (Vanderwinden 1996b; Ekblad 1998; Horisawa 1998; Yamataka 1998; He 2000).

In the present study we combined histochemistry and immunohistochemistry to examine anatomical relationship between nitrergic neuronal network and c-kit positive cellular network in the normal human bowel using whole-mount preparation technique. This technique clearly demonstrated three-dimensional morphology of c-kit positive cellular networks and nitrergic nerves in detail. The distribution of c-kit positive cellular network and nitrergic neuronal network in the muscle layer of bowel was consistent with the previously reported findings (Faussone-Pellegrini 1990; Rumessen 1993; Sanders 1996; Yamataka 1996; Yamataka 1998). The three dimensional network formed by c-kit positive cells was located between the circular and longitudinal muscle layers of the bowel wall, at the innermost part of the circular muscle layer and within the circular muscle layer. The whole-mount preparation elegantly demonstrated nitrergic neuronal network in the myenteric plexus as mesh of nerve fibers with ganglia, containing cluster of ganglion cells between the two layers of the bowel wall.

The association between the c-kit positive cellular network and nitrergic neuronal network was clearly demonstrated by double-labelling histochemistry and immunohistochemistry and by double immunofluorohistochemistry using confocal laser scanning microscopy. Close association was observed between the c-kit positive cellular network and nitrergic neuronal network. The myenteric plexus was surrounded by a dense reticular network of c-kit positive cells. Although the c-kit positive cellular network was closely apposed to and intermingled freely with the myenteric plexus, no evidence indicating direct contact between the two networks could be found either by confocal laser scanning microscopy or by double staining with NADPH-diaphorase histochemistry and c-kit immunohistochemistry. The relationship between nerves and ICC remains unclear. In S_l/S_{ld} mice that do not produce membrane bound stem cell factor, the ligand for kit, small intestinal ICC at the level of the myenteric plexus are absent, suggesting that the stem cell factor is necessary for their development. The major source of stem cell factor is enteric neurons. This would suggest that primary deficit in enteric neurons secreting stem cell factor results in loss or a decreased in the number of ICCs. However, in contrast W/W^l mutant mice that lack ICCs in the small intestine and have absent small intestinal electrical slow waves and abnormal uncoordinated motility have normal myenteric plexus, suggesting that ICC are not required for the development or maintenance of myenteric plexus (Malys 1996).

The development of double NADPH-diaphorase histochemistry and c-kit immunohistochemistry staining technique in whole mount preparation provides an easy and useful method for investigating c-kit positive cellular network and nitrergic neuronal network in the human bowel wall. In the present study this technique demonstrated close association between these two networks. Since the dense reticular network of ICCs intimately surrounds the nitrergic neuronal plexus and have abundant gap junctions connected with smooth muscle cells and nerve fibres, it is very likely that they play a role as mediator in neurotransmission, possibly the non-adrenergic, non-cholinergic inhibition. There are a number of intestinal motility disorders in which defects in innervation and loss or structural alteration of ICC have been reported, *e.g.* hypertrophic pyloric stenosis, HD, intestinal pseudo-obstruction and slow-transit constipation. The characteristic profiles of c-kit positive cellular network and nitrergic neuronal network observed in this study provide a morphological basis for investigating intestinal motility disorders.

It is currently accepted that the cellular network of ICCs is connected by gap junctions (Thuneberg 1989; Sanders

1996; Nemeth 2000a). Although the crucial role of gap junctions in the intercellular communication in the gut musculature has been shown by the diffusion experiment using neurobiotin (Farraway 1995), there have been no studies that have revealed the distribution of gap junction protein in the muscular coat in the human colon and also in the ICCs. The contraction relaxation sequence of the smooth muscle cells is a direct consequence of the cyclic depolarisation and repolarisation of membranes of the muscle cells. Gap junctions are sites of propagation or conduction of action potentials between cells of almost all tissues (Garfield 1995; Morley 1997). Gap junctions are intercellular channels that link cells to their neighbours and allow current carrying inorganic ions and small molecules to pass between cells, thereby facilitating electrical and metabolic coupling. The pores of the gap junction channels, which connect the interiors of two cells, are composed of connexins. Connexin43 is reported to be the most important gap junction protein in humans (Mikkelsen 1993; Lau 1996; Nagaoka 1999). By using a type-specific antibody against connexin43, we have demonstrated a unique localisation of gap junction protein connexin43. Double immunostaining revealed that connexin43 expression was present in the cell body and processes of the c-kit positive ICCs. The expression of connexin43 protein is abundant in the ICCs in normal bowel. In the innermost layer of the circular muscle, and between the two muscle layers where the ICCs form a three-dimensional network, strong connexin43 expression was present in the cytoplasm of ICCs and between the ICCs and the smooth muscle fibres. Confocal laser scanning microscopy clearly demonstrated that the gap junction protein connexin43 was present in the circular muscle fibres probably where the ICCs make contact with the muscle fibres.

Some studies have suggested that gap junction channel expression or regulation is altered in various disease states (Garfield 1995; Lau 1996; Mambetisawa 1999; Nemeth 2000a). Changes in gap junction channel composition, or on the levels of connexin expression, have been proposed as determinants for the ability of action potentials to propagate across the neighbouring cells. Immunohistochemical studies have shown that the expression of gap junction channel proteins decreases in the areas affected by myocardial infarction (Lau 1996). Other investigations have shown that chronic exposure of cardiac cells to cAMP can change the level of expression of individual connexins (Morley 1997).

Gap junctions play important roles as sites for the coordination of action of substances, which either stimulate or inhibit the smooth muscle cells to contract. The propagation of electrical signals is key to control of contractility. Lack of conducted action potentials and inactivity of the smooth muscle cells are promoted by decreases in pacemaker activity, excitability of muscle cells and cell-to-cell coupling. Any mechanism that suppresses the generation of action potentials, closes gap junctions, or decreases their numbers inhibits the active events and thereby affects contractility. The lack of expression of connexin43 in the circular muscle layer and between the circular and longitudinal muscle layers in the aganglionic bowel and decreased connexin43 expression in the transitional zone suggest that the intercellular signalling is impaired in Hirschsprung's disease. Lack of gap junctions in HD may prevent exchange of hormones, neurotransmitters and other antagonists between ICCs and smooth muscle cells and thereby level of ability of action potentials to propagate, causing motility dysfunction. Cellular networks of pacemaker activity in intestinal motility are still matter of debate. The hypothesis that gap junctions represent an additional pathway for homotypic and heterotypic cell-cell interactions in the gastrointestinal tract is truly exciting. More connexins such as Cx26, Cx32 and Cx45 are surely involved in the nervous system of bowel under physiological and pathological conditions. Future studies on gap junctional intercellular communication in the bowel wall may provide important clues for the understanding of regulatory mechanism of intestinal motility. These studies range the human motility disorders, genetically induced mouse aganglionosis and the changes of the gap junctional proteins in chemically induced segmental aganglionosis. Specific antibodies and molecular probes for distinct connexins are now available, such that they can be identified by immunocytochemistry, immunoblot and molecular biology strategies. However, functional analysis of gap junction mediated cell-cell communication requires further methodoligical approaches that comprise metabolic cooperation, injection of low-molecular weight dyes and standard electrophysiological techniques.

References

Bult H, Boeckxstaens GE, Pelckmans PA (1990) Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345:346-347.

Delorme R, Benchaib M, Bryon PA (1998) Measurement accuracy in confocal microscopy. J Microscopy 192:151-157.

Desai KM, Sessa WC, Vane JR (1991) Involvement of nitric oxide in the reflex relaxation of the stomach to accommodate food or fluid. Nature 351:477-479.

Ekblad E, Sjuve R, Arner A (1998) Enteric neuronal plasticity and a reduced number of interstitial cells of Cajal in hypertrophic rat ileum. Gut 42:836-844.

Farraway L, Ball AK, Huizinga JD (1995) Intercellular metabolic coupling in canine colon musculature. Am J Physiol 268:C1492-1502.

Faussone-Pellegrini MS, Pantalone D, Cortesini C (1990) Smooth muscle cells, interstitial cells of Cajal and myenteric plexus interrelationships in the human colon. Acta Anat 139:31-44.

Fekete E, Resch BA, Benedeczky I (1995) Histochemical and ultrastructural features of the developing enteric nervous system of the human foetal small intestine. Histol Histopathol 10:127-134.

Ferri GL, Botti PL, Vezzadini P (1982) Peptide-containing innervation of the human intestinal mucosa. An immunocytochemical study on whole-mount preparations. Histochemistry 76:413-420.

Garfield RE, Ali M, Yallampalli C (1995) Role of gap junctions and nitric oxide in control of myometrial contractility. Semin Perinatol 19:41-51.

Hagger R, Gharaie S, Finlayson C (1998) Distribution of the interstitial

cells of Cajal in the human anorectum. J Auton Nerv Syst 73:75-79.
He CL, Burgart L, Wang L (2000) Decreased interstitial cell of Cajal volume in patients with slow-transit constipation. Gastroenterol 118:14-21.
Horisawa M, Watanabe Y, Torihashi S (1998) Distribution of c-Kit immunopositive cells in normal human colon and in Hirschsprung's disease. J Pediatr Surg 33:1209-1214.
Lau AF, Kurata WE, Kanemitsu MY (1996) Regulation of connexin43 function by activated tyrosine protein kinases. J Bioenerg Biomembr 28:359-368.
Li Z, Zhou Z, Daniel EE (1993) Expression of gap junction connexin 43 and connexin 43 mRNA in different regional tissues of intestine in dog. Am J Physiol 265:G911-916.
Maeda H, Yamagata A, Nishikawa S (1992) Requirement of c-kit for development of intestinal pacemaker system. Development 116:369-375.
Malysz J, Thuneberg L, Mikkelsen HB (1996) Action potential generation in the small intestine of W mutant mice that lack interstitial cells of Cajal. Am J Physiol 271:G387-399.
Mambetisaeva ET, Gire V, Evans WH (1999) Multiple connexin expression in peripheral nerve, Schwann cells, and Schwannoma cells. J Neurosci Res 57:166-175.
Mebis J, Penninckx F, Geboes K (1990) Neuropathology of Hirschsprung's disease: en face study of microdissected intestine. Hepatogastroenterol 37:596-600.
Mikkelsen HB, Huizinga JD, Thuneberg L (1993) Immunohistochemical localization of a gap junction protein (connexin43) in the muscularis externa of murine, canine, and human intestine. Cell Tissue Res 274:249-256.
Morley GE, Ek-Vitorin JF, Taffet SM (1997) Structure of connexin43 and its regulation by pH. J Cardiovasc Electrophysiol 8:939-951.
Nagaoka T, Oyamada M, Okajima S (1999) Differential expression of gap junction proteins connexin26, 32, and 43 in normal and crush-injured rat sciatic nerves: Close relationship between connexin43 and occludin in the perineurium. J Histochem Cytochem 47:937-948.
Nakamura K, Kuraoka A, Kawabuchi M (1998) Specific localization of gap junction protein, connexin45, in the deep muscular plexus of dog and rat small intestine. Cell Tissue Res 292:487-494.
Nemeth L, Fourcade L, Puri P (2000a) Marked morphological differences in the myenteric plexus between the mesenteric and antimesenteric sides of small bowel in premature infants. J Pediatr Surg 35:748-752.
Nemeth L, Maddur S, Puri P (2000b)Immunolocalization of the gap junction protein connexin43 in the interstitial cells of Cajal in the normal and Hirschsprung's disease bowel. J Pediatr Surg 35:823-828.
Puri P (1993) Hirschsprung's disease: clinical and experimental observations. World J Surg 17:374-384.
Puri P, Ohshiro K, Wester T (1998) Hirschsprung's disease: a search for etiology. Semin Pediatr Surg 7:140-147.
Puri P (1997) Hirschsprung disease. In Oldham KT, Colombani PM, Foglia RP, eds., Surgery in Infants and Children: Scientific Principles and Practice, Lippincott-Raven Publishers, Philadelphia, pp. 1277-1290.
Rumessen JJ, Peters S, Thuneberg L (1993) Light- and electron microscopical studies of interstitial cells of Cajal and muscle cells at the submucosal border of human colon. Lab Invest 68:481-495.
Rumessen JJ, Thuneberg L (1996) Pacemaker cells in the gastrointestinal tract: interstitial cells of Cajal. Scand J Gastroenterol 216:82-94.
Sanders KM (1996) A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. Gastroenterol 111:492-515.
Simon AM (1999) Gap junctions: more roles and new structural data. Trends Cell Biol 9:169-170.
Thuneberg L (1989) Interstitial cells of Cajal, In Wood JD, ed., Handbook of Physiology. The gastrointestinal system, Vol.1, Am Physiol Soc, Bethesda MD, pp. 349-386.
Toma H, Nakamura K, Kuraoka A (1999) Three-dimensional structures of c-Kit-positive cellular networks in the guinea pig small intestine and colon. Cell Tissue Res 295:425-436.
Torihashi S, Ward SM, Nishikawa S (1995) c-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res 280:97-111.
Xue C, Pollock J, Schmidt HH (1994) Expression of nitric oxide synthase immunoreactivity by interstitial cells of the canine proximal colon. J Auton Nerv Syst 49:1-14.
Yamataka A, Fujiwara T, Nishiye H (1996) Localization of intestinal pacemaker cells and synapses in the muscle layers of a patient with colonic hypoganglionosis. J Pediatr Surg 31:584-587.
Yamataka A, Ohshiro K, Kobayashi H (1997) Intestinal pacemaker C-KIT+ cells and synapses in allied Hirschsprung's disorders. J Pediatr Surg 32:1069-1074.
Yamataka A, Ohshiro K, Kobayashi H (1998) Abnormal distribution of intestinal pacemaker (C-KIT-positive) cells in an infant with chronic idiopathic intestinal pseudoobstruction. J Pediatr Surg 33:859-862.
Vanderwinden JM, Liu H, Menu R (1996) The pathology of infantile hypertrophic pyloric stenosis after healing. J Pediatr Surg 31:1530-1534.
Vanderwinden JM, Rumessen JJ, Liu H (1996) Interstitial cells of Cajal in human colon and in Hirschsprung's disease. Gastroenterol 111:901-910.
Wedel T, Krammer HJ, Kuhnel W (1998) Alterations of the enteric nervous system in neonatal necrotizing enterocolitis revealed by whole-mount immunohistochemistry. Pediatr Pathol Lab Med 18:57-70.
Wester T, O'Briain DS, Puri P (1999a) Notable postnatal alterations in the myenteric plexus of normal human bowel. Gut 44:666-674.
Wester T, Eriksson L, Olsson Y (1999b) Interstitial cells of Cajal in the human fetal small bowel as shown by c-kit immunohistochemistry. Gut 44:65-71.

Volume 45(1-4):65-73, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Novel carbon fiber microeletrodes for extracellular electrophysiology

Dénes Budai[1]*, Zsolt Molnár[2]

[1]Department of Biology, Juhász Gyula College, University of Szeged, Szeged, Hungary, [2]Department of Medical Chemistry Faculty of Medicine, University of Szeged, Szeged, Hungary

ABSTRACT Single- and multibarrel carbon fiber microelectrode blanks were constructed and pulled to electrodes to be used for extracellular recording and microiontophoresis. A unique spark etching method was developed to produce a sharp-pointed, conical carbon tip protruding 15-20 μm from the glass pipette(s). The shape and size of the carbon fiber tip were examined by scanning electron microscopy. In test experiments, extracellular recordings were made from spinal dorsal horn neurons of the spinal cord in anesthetized rats. The sharp carbon tip allowed these electrodes to penetrate the arachnoid membrane over the spinal cord with ease. The electrodes picked up extracellular spikes with an excellent signal-to-noise ratio. Under the given experimental conditions, the peak-to-peak noise level was about 20 μV. To test the performance of the iontophoresis barrels, neurons were stimulated by iontophoretic application of *N*-methyl-D-aspartate (NMDA) or kainic acid or by noxious heat delivered to the cutaneous receptive fields in the tail. After the iontophoretic ejection of naloxone, the responses to iontophoresed kainic acid and noxious heat were significantly increased. Spikes from dorsal horn neurons were counted and peristimulus time histograms were displayed on-line by means of a LabView-based system. These carbon fiber microelectrodes are excellent for extracellular spike recording and microiontophoresis and may additionally be suitable for electrochemical measurements and for the development of enzyme- or antibody-based microbiosensors.

Acta Biol Szeged 45(1-4):65-73 (2001)

KEY WORDS

multibarrel microelectrodes
carbon tip
spark etching
scanning electron microscopy
single-unit recording
microiontophoresis
electrochemistry
microbiosensors

Carbon fiber microelectrodes have been used to record extracellular action potentials since 1979 (Armstrong-James and Millar 1979; Armstrong-James et al. 1980a; Anderson and Cushman 1981). The carbon fibers are graphite monofilaments about 7 μm in diameter. In microelectrodes, they have good extracellular recording qualities similar to those of the best tungsten electrodes (Fox et al. 1980; Shigemitsu et al. 1980; Starrenburg and Burger 1982; Yavich 1998). These microelectrodes have been demonstrated to be suitable for *in vivo* electrochemical detection (Ponchon et al. 1979; Armstrong-James et al. 1980b; Millar et al. 1981; Stamford et al. 1984; Millar et al. 1992). Various voltammetric or amperometric techniques involving the use of carbon fiber microelectrodes have been developed for *in vivo* measurements of biogenic amines (Kruk et al. 1980; Buda et al. 1981; Cespuglio et al. 1981; Gonon et al. 1981; Akiyama et al. 1985; Rivot et al. 1987; Mermet et al. 1990; Kawagoe et al. 1991b; Suaud-Chagny et al. 1993; Rivot et al. 1995; Shigenaga et al. 1997; Daws et al. 1998) or nitric oxide (Malinski and Tah 1992; Yao et al. 1995; Friedemann et al. 1996; Park et al. 1998; Clarencon et al. 1999). Carbon fiber-based, enzyme-modified microbiosensors have been introduced for the *in situ* determination of glucose (Netchiporouk et al. 1996; Shram et al. 1997), acetylcholine (Kawagoe et al. 1991a; Navera et al. 1991; Tamiya and Karube 1992), choline (Garguilo and Michael 1996), lactate (Shram et al. 1998) or glutamate (Kulagina et al. 1999).

During the fabrication of such microelectrodes, individual carbon fibers can be inserted into borosilicate glass capillary tubing and single or multibarrel electrode blanks can easily be assembled. Because of the great tensile strength of the carbon fibers, they do not break when blanks are pulled to microelectrodes. After the pulling, the microelectrode is left with several centimeters of carbon fiber protruding from the glass tip. The simplest way to trim the end of the carbon fiber to the correct tip length (10-30 μm) is to cut off the excess with microscissors under a microscope. This is a difficult operation even for an experienced worker with steady hands, and the glass tip can easily be damaged. Another method of trimming the carbon fiber is electrochemical etching, applying dilute chromic acid (Millar and Williams 1988) or saline (Kuras and Gutmaniene 1995; Fu and Lorden 1996; Kuras and Gutmaniene 2000) and a few tenths of a mA of alternating current. A third technique is spark etching, which allows the best control of tip length and shape for selective

Accepted May 17, 2001
*Corresponding author. E-mail: budai@jgytf.u-szeged.hu

extracellular unit recording or electrochemical measurements. In the present study we have developed a relatively simple method of carbon fiber microelectrode manufacturing, which uses spark etching to form a sharp-pointed conical tip with a length of 15-20 μm.

Materials and Methods

Manufacturing carbon fiber electrodes

Carbon fiber electrodes were fabricated from borosilicate glass capillary tubing obtained from World Precision Instruments (WPI, Sarasota, FL, USA). Single-barrel, recording-only carbon fiber electrodes were made from standard borosilicate glass tubing (1.50 mm o.d., 0.84 mm i.d.). Multibarrel recording/iontophoresis electrodes were constructed from the appropriate number of thin-wall glass tubes (1.50 mm o.d., 1.12 mm i.d.) glued together before pulling. The recording barrel contained no inner filament, whereas the iontophoresis barrels were made from glass tubing with a solid inner glass filament fused to the inner wall, which accelerates the filling of the barrels. A 15 cm long individual carbon fiber with a diameter of about 7 μm was glued to a 2.5 cm long 28 AWG tin-plated copper wire with conductive paint (Silver print, GC Electronics, Rockford, IL, USA). One end of the wire had previously been soldered into a gold-plated male connector pin. Beginning at its free end, the carbon fiber was sucked into the glass capillary tubing by gentle vacuum. The connector pin was then fixed onto the end of the glass tubing by 12 mm long heat-shrinkable plastic tubing. For single electrodes, this assembly was ready to be pulled. For multibarreled arrays, the appropriate number of inner filament-containing capillary tubes were attached to the recording barrel with two-component epoxy glue at both ends of the arrays. The glued portions of the arrays were covered with 15 mm long heat-shrinkable plastic tubing at both ends to provide further stability for the arrangement and suitable locations for keeping the multibarrels in place during pulling and in the electrode holder.

The two ends of the electrode "blank" were then held by the chucks of the vertical electrode puller (PE-2, Narishige Scientific, Tokyo, Japan) and a heating coil was used to melt the glass gently in the central portion of the assembly. As the glass was beginning to soften, the lower chuck was slowly rotated by one-half to two-thirds of a full circle while, the electrode blank was pulled slowly by gravity only. This rotation and pulling caused the lengths of tubing to fuse together. The combination of the current supply to the heating coil and the degree and timing of the pull may be varied to produce pipettes of different lengths and diameters. In consequence of the very high tensile strength of the carbon fiber, it did not break during the pulling procedure. The excess fiber protruding from the tip of the glass assembly was shortened with fine scissors to about 5 mm. The exposed carbon fiber was finally trimmed by spark etching (Millar 1992; Williams et al. 1992a,b) under a light microscope. Sparks were generated by a high voltage of about 800 V, with a piece of polished gold wire as counter electrode. Finally, the open ends of the glass tubing were heated up and bent out radially from the center to facilitate access and to reduce cross-contamination between barrels during filling.

Scanning electron microscopy

Electrode samples for scanning electron microscopy were coated with conductive films of gold with the aid of a sputter coater (SC-520, Bio-Rad, Hercules, CA, USA). Images of electrode tips were taken with a traditional scanning electron microscope (S-2400, Hitachi, Tokyo, Japan).

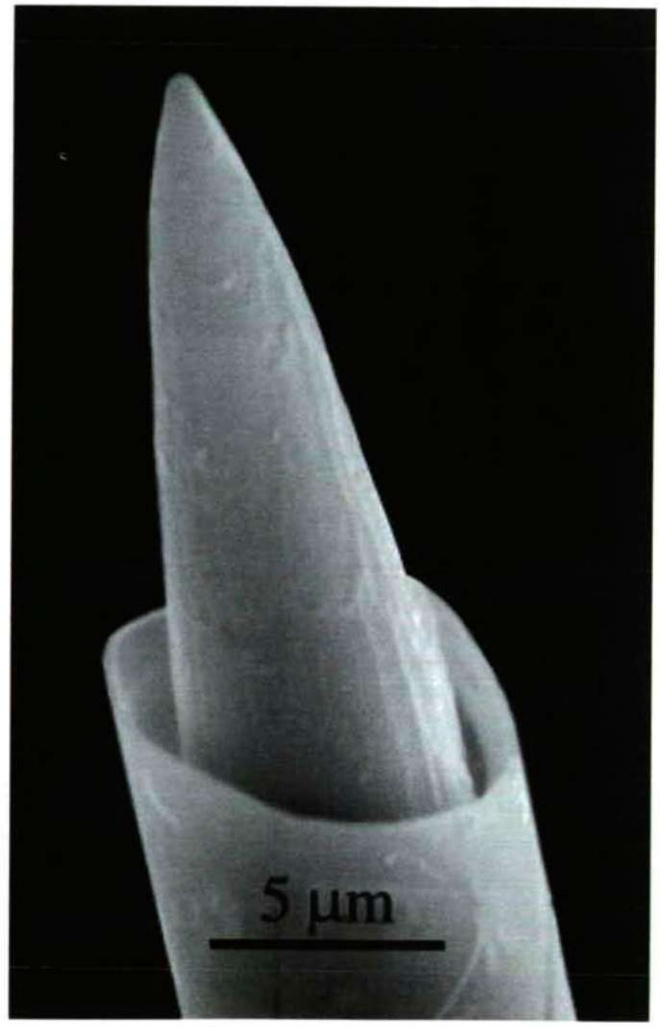


Figure 1. Scanning electron micrograph of the tip of a single-barrel carbon fiber electrode. The sharp-pointed, conical tip of the carbon fiber protrudes from the insulating borosilicate glass capillary tubing. The carbon tip is suitable for extracellular recording and for *in vivo* electrochemical measurements.

Anesthesia and surgery

Sprague-Dawley rats (300-400 g) of either sex were initially anesthetized with chloral hydrate (40 mg/100 g, *i.p.*). For single-unit recordings, the sacral spinal cord was exposed by a laminectomy and the rat was placed in a stereotaxic apparatus. The spinal cord was covered with a pool of warmed mineral oil. Body temperature was kept at 37°C by a water-heated blanket beneath the rat and an infrared heat lamp from above. Heart rate was monitored and maintained within normal limits for lightly anesthetized rats. Recordings were commenced not earlier than one hour after surgery. During the experiments, the animals were maintained in a lightly anesthetized state with additional *i.p.* injections of chloral hydrate, so that the rats showed no sign of discomfort, but the tail flick reflex could be evoked by the application of noxious heat (43-45°C) to the tail.

Extracellular recording

Extracellular, single-unit recordings were made from neurons of the dorsal horn of the sacral spinal cord. Recording/iontophoresis electrodes were constructed from a seven-barreled array of borosilicate glass capillary tubing as described above. Drug solutions were filled into the free ends of the iontophoresis barrels, and capillary action in the inner glass filaments ensured complete filling. The tip of the electrodes was kept in physiological saline during the filling procedure in order to prevent cross-contamination between the barrels and to prevent drying-out of the tip. Drugs were delivered iontophoretically using nanoampere currents of appropriate polarity. Action potentials were recorded with an ExAmp-20KB amplifier (Kation Scientific, Minneapolis, MN, USA) and displayed on an oscilloscope, and the activity of single units was isolated by using a window discriminator. The collection of experimental data and the iontophoretic delivery of drugs were automated by means of a multifunction instrument control and data acquisition board (PC-1200, National Instruments, Austin, TX, USA) programmed in

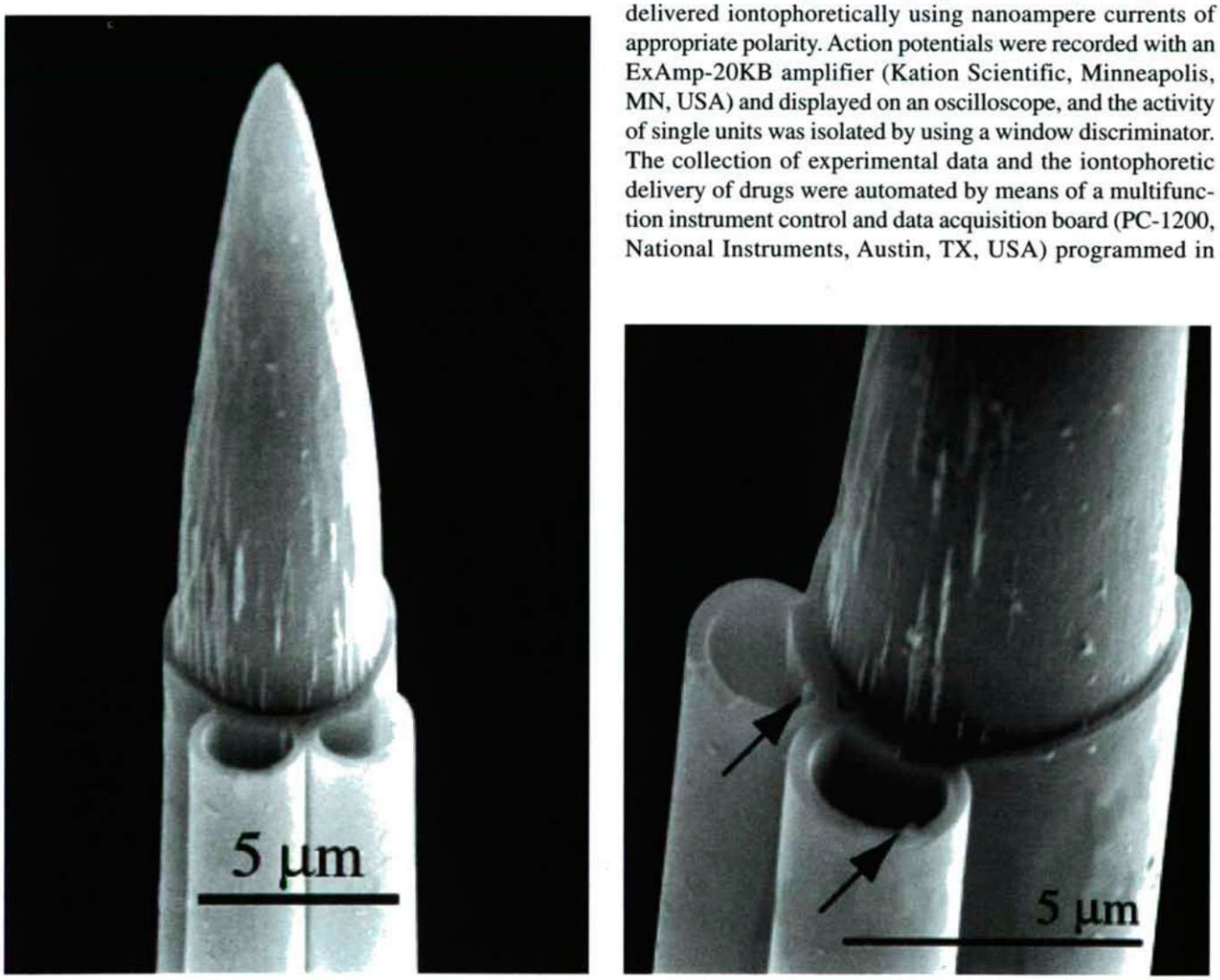


Figure 2. Fine structure of the tip of a three-barreled carbon fiber combination electrode as revealed by scanning electron microscopy. Left panel: the conducting carbon fiber tip records electrical signals; microiontophoresis can be performed through the microscopic orifices of the side-positioned iontophoresis barrels. Right panel: close-up view of the iontophoresis barrels. Note the internal glass filaments (arrows) that permit self-filling of aqueous solutions through the free ends of the capillary tubing.

LabView (National Instruments). LabView-based data acquisition and instrument control software was developed in-house; it has been published in part elsewhere (Budai 1994, 2000). Histograms of the neuronal activity were taken on-line by the computer-controlled system. Analog signals were sampled and digitized at 20 kHz by the data acquisition card, and oscilloscope trace-like recordings were taken from neurons at the beginning of each experiment.

Single-unit extracellular recordings were made from selected dorsal horn neurons responding to noxious heat delivered by a projector lamp focused on the blackened ventral surface of the tail. A thermistor probe placed in contact with the heated area was used to provide feedback control of the heat stimulus in order to prevent overheating. The temperature was raised to a peak of 50°C and held there for 5 s. Neurons were also characterized as low threshold (LT), nociceptive specific (NS) or wide dynamic range (WDR) according to their responses to mechanical stimuli of increasing strength. Both innocuous (brush, pressure) and noxious (pinch, squeeze felt to be painful by the experimenter) stimuli were applied to the excitatory receptive fields of the tail.

Microiontophoresis and drugs

Microiontophoresis was performed with Minion-16 iontophoresis current units (Kation Scientific, Minneapolis, MN, USA). The drug barrels of the combined seven-barreled recording/iontophoresis electrode contained one or other of the following freshly made solutions: 100 mM *N*-methyl-D-aspartate Na (NMDA) in 100 mM NaCl (pH 8.0), 20 mM kainic acid (KA) in 180 mM NaCl (pH 8.0), or 20 mM naloxone HCl in 180 mM NaCl (pH 5.0). NMDA and KA were delivered by negative currents, while naloxone was ejected by positive current. All drugs, including chloral hydrate, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Results

Scanning electron microscopy

Scanning electron microscopy was used to inspect the shape and size of the carbon fiber tips trimmed by spark etching.

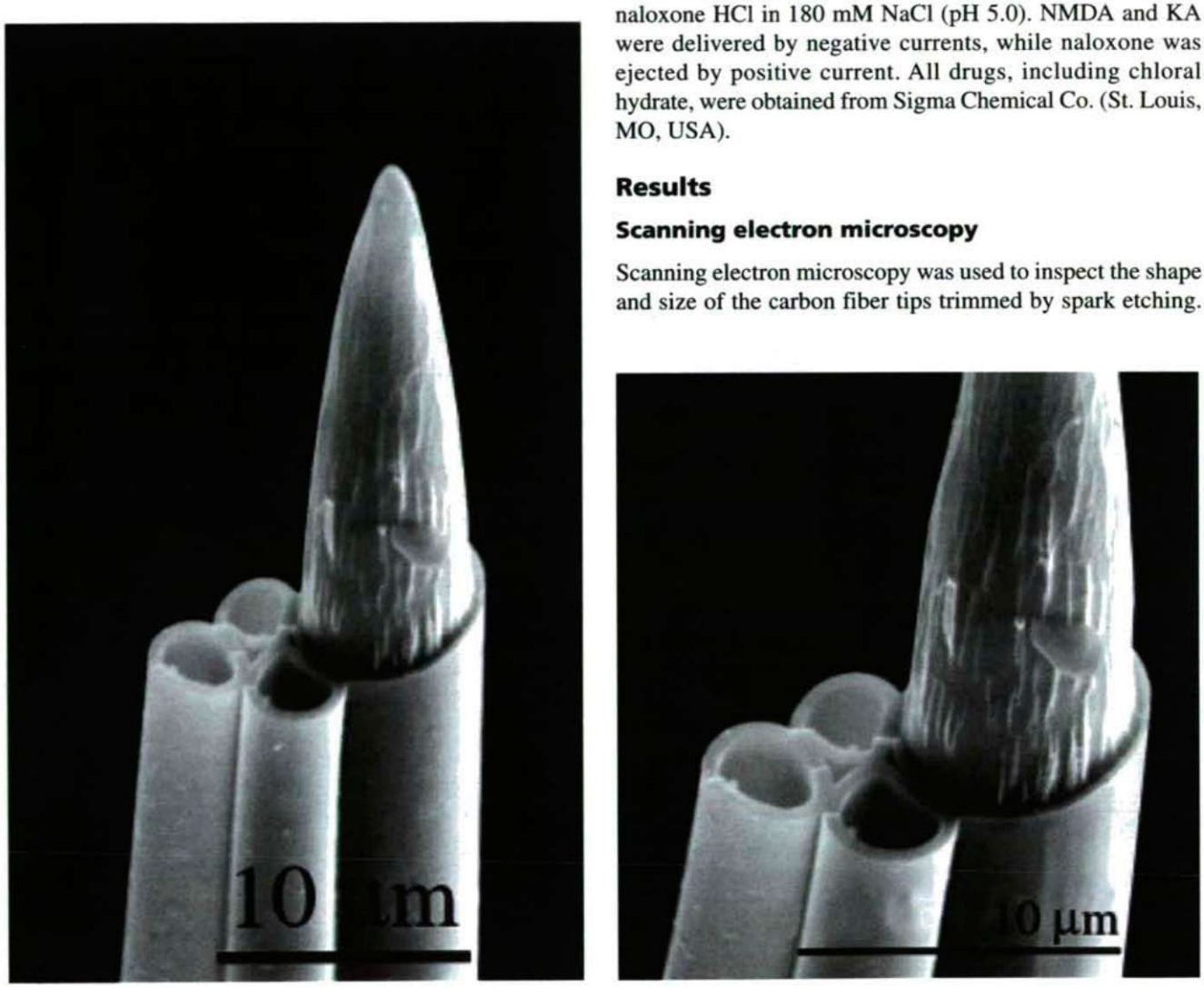


Figure 3. The four-barreled carbon fiber combination electrode allows the testing of three neuroactive compounds simultaneously. The carbon tip records extracellular spikes. Left panel: scanning electron microscopic image of the tip of a four-barreled electrode. Right panel: close-up view of the iontophoresis barrels.

As Figs. 1-4 show, this procedure resulted in characteristic, conical carbon tips that extended about 15 µm from the glass pipettes. Single-barrel electrodes were made for extracellular action potential recording and/or electrochemical measurements. The borosilicate glass wall around the carbon fiber provided the necessary electrical insulation (Fig. 1). In multibarrel assemblies, the tips of the iontophoresis barrels were very much smaller than the carbon fiber barrel. The spark etching of the carbon fiber in multibarrel microelectrodes was completed in the same way as for single electrodes, and their recording qualities were directly comparable with those of the single electrodes. Figure 2 shows a scanning electron micrograph of a typical three-barreled microelectrode. The glass insulation around the fiber ends is at the same level as the iontophoresis barrels. Three barrels are suitable when iontophoresis is used only to mark the recording site with iontophoresed tracer material or when one substance (or possibly two) is to be tested on neuronal firing. When a greater number of drugs are to be tested by microiontophoresis in combination with extracellular spike recording, four- or seven-barrel constructions should be used (Figs. 3 and 4, respectively). Electron microscopy revealed that in asymmetric arrangements of carbon fiber and iontophoresis barrels, such as in the three- and four-barrel electrodes, the iontophoresis barrels ended in oval or moon-shaped apertures (Figs. 2 and 3). In contrast, the symmetric construction of the seven-barrel electrodes resulted in six trapezoid-like iontophoresis orifices around the center barrel that contained the carbon fiber (Fig. 4). In some of the electrodes, however, the carbon fiber was pushed laterally away from the center position during the pulling process, which led to the formation of uneven iontophoresis openings (Fig. 4).

In vivo experiments

The recording quality of the carbon fibers and the performance of the microiontophoresis barrels were examined in experiments on anesthetized rats. Extracellular recordings were made from spinal dorsal horn neurons located between 100 and 600 µm from the dorsal surface of the spinal cord. The sharp carbon tip allowed these electrodes to penetrate the arachnoid membrane over the spinal cord with ease. In a few experiments, the electrodes successfully perforated even the dura mater over the rat spinal cord and extracellular recordings were possible. Measured in physiological saline at 1 kHz, the impedances of these electrodes lay between 400 KΩ and 1 MΩ. There was no consistent relationship between impedance and tip length. Within the nervous tissue, the electrodes picked up extracellular spikes with an excellent signal-to-noise ratio. Under the experimental conditions applied, when 30 Hz to 8 KHz filters were used, the peak-

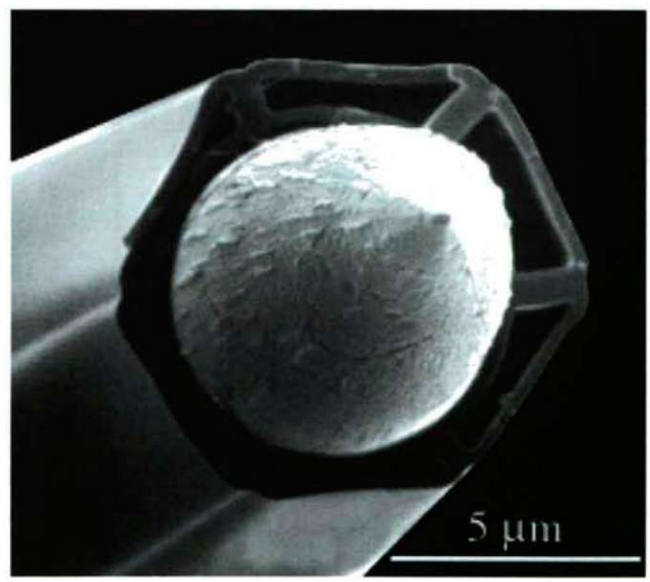


Figure 4. The seven-barreled carbon fiber combination electrode allows the testing of up to six neuroactive compounds on neuronal firing recorded by the carbon tip. Left panel: the fused-together glass micropipettes form a seal around the protruding carbon fiber. Right panel: close-up image of the iontophoresis barrels and the axial carbon fiber tip.

to-peak noise level was about 20 μV (Fig. 5). The impedances of iontophoresis barrels filled with 200 mM NaCl and measured at 1 kHz varied between 2 MΩ and 12 MΩ. The differences in impedance between the iontophoresis barrels of the seven-barreled electrodes were greater than those for the three- or four-barrel electrodes.

In sample experiments, the sensory neurons of the dorsal horn were stimulated by iontophoretic applications of NMDA and/or KA or by noxious heat delivered to the cutaneous receptive fields in the tail. Spike recordings and microiontophoresis of drugs were performed with seven-barreled carbon fiber electrodes. A sample record tracing of NMDA-evoked spikes is shown in Fig. 5. Spikes from dorsal horn neurons were counted by the data acquisition board and peristimulus time histograms were displayed on-line by means of the LabView-based system (Fig. 6). The effects of iontophoresed naloxone on the responses evoked by the application of iontophoresed NMDA or kainic acid or of peripheral noxious heat in a low threshold (LT) dorsal horn neuron are shown in Fig. 6. After the iontophoretic ejection of naloxone, the responses to iontophoresed kainic acid and noxious heat were significantly increased. In contrast, the responses to iontophoretically applied NMDA were markedly decreased (Fig. 6). With either single- or multibarreled carbon fiber electrodes, the quality of the spike recordings could be maintained for many hours.

Discussion

Single- or multibarrel carbon fiber microelectrodes are used to record extracellular action potentials. Extracellular "spikes" are typically a few hundred microvolts in amplitude and are generated by action potentials across the membranes of neurons. Extracellular recording of neuronal firing is often used in conjunction with the microiontophoresis of various neuroactive compounds (Hicks 1984). This requires compound recording/iontophoresis multibarrel microelectrode

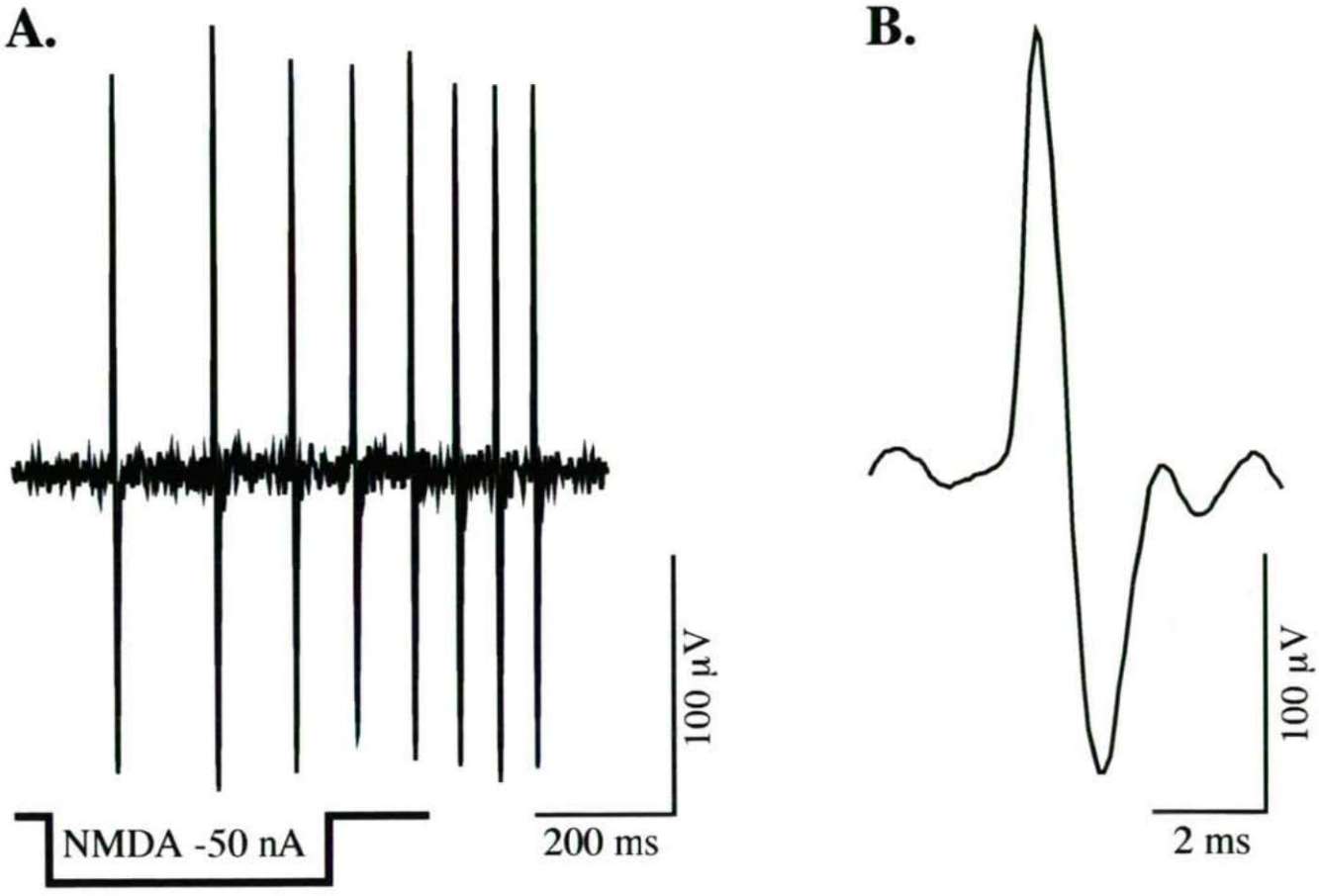


Figure 5. Sample spike records from a low threshold (LT) spinal dorsal horn neuron, using a seven-barreled carbon fiber recording/iontophoresis electrode. **A.** Neuronal firing was evoked by NMDA iontophoresis as shown. **B.** Shape of an individual extracellular action potential. Note the excellent signal-to-noise ratio recording achieved with the carbon fiber electrode.

assemblies capable both of leading electrical signals into the preamplifier and of delivering test compounds into the near vicinity of neurons, using electrical currents in the nanoampere range. The basic multibarrel micropipette assembly was introduced by D. R. Curtis (Curtis 1964). Neuronal spikes can be recorded through a glass micropipette if filled with a suitable electrolyte solution such as sodium chloride. However, electrolyte-filled micropipettes in a multibarrel assembly are electrically very "noisy". The solid-conductor microelectrodes such as the tungsten or carbon fiber electrodes, in contrast, exhibit significantly less noise in extracellular recordings. For this reason, many attempts have been made to combine a tungsten electrode with a set of iontophoresis barrels. The usual technique has been to glue the two types of electrodes together (piggyback configuration; Krnjevic 1971) or to insert a presharpened tungsten wire into one of the barrels (metal-in-glass configuration; Kasser and Cheney 1983; Hellier et al. 1990; Li et al. 1990; Godwin 1993; Haidarliu et al. 1995). Either method involves a technically very difficult and time-consuming procedure.

The carbon fiber-containing recording/microiontophoresis combination electrodes are considerably easier and cheaper to make than tungsten electrode-containing multibarrel assemblies. The single most difficult task in making carbon fiber microelectrodes is to attain the correct length and shape of the carbon tip protruding from the glass micropipettes. In our experience, cutting with fine scissors proved very tedious and the rate of successfully finished electrodes was low. Etching by means of an alternating current in a drop of dilute chromic acid or physiological saline proved to be a more reliable method and produced better carbon tips. However, the tip of a finished microelectrode must be kept in distilled water overnight in order to remove the acid or salt contamination from the iontophoresis barrels. As a rule, once the iontophoresis barrels have been filled with any aqueous solution, the tip must never be allowed to dry out. Otherwise, the microcrystals formed damage the fine glass configuration in the tip and the electrode becomes useless due to the production of excessive electrical noise. To circumvent these problems, we introduced and improved the spark etching method to achieve sharp-pointed conical carbon tips protruding 15-20 µm from the glass assembly. Since no solutions are involved, this dry method means that such microelectrodes may be stored in a dust-free place for many weeks without loss of their good recording qualities. In consequence of the inner glass filaments, the iontophoresis barrels are simple to fill, and the sharp tip penetrates tissues with ease. These carbon tips may additionally be suitable for electrochemical measurements and for the development of enzyme- or antibody-based microbiosensors (Kawagoe et al. 1991a; Garguilo and Michael 1996; Netchiporouk et al. 1996; Darbon et al. 1998).

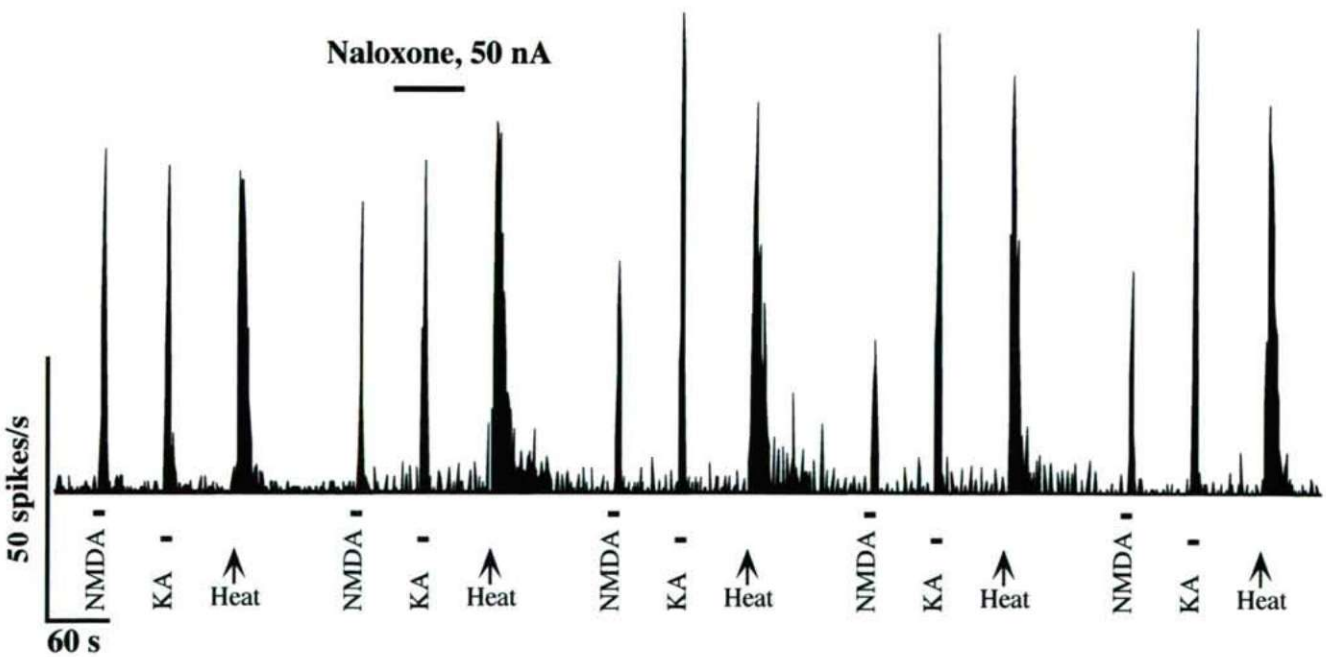


Figure 6. Ratemeter recordings showing the effects of iontophoretically applied naloxone on a wide dynamic range (WDR) dorsal horn neuron excited by iontophoresed NMDA (ejected at -52 nA for 5 s) and KA (-15 nA, 5s) and noxious heat stimulation of the cutaneous receptive field. Drugs were ejected as indicated by the horizontal bars. Note the facilitating effect of naloxone on the KA- and heat-evoked responses. In contrast, the responses to NMDA were inhibited in the presence of naloxone.

Acknowledgments

We thank Dr. Erzsébet Mihalik and the Electron Microscopy Laboratory of the Department of Botany, University of Szeged, for taking the scanning electron micrographs. The technical assistance of Mr. Ferenc Tarjányi in the experimental work is also gratefully acknowledged.

References

Akiyama A, Kato T, Ishii K, Yasuda E (1985) In vitro measurement of dopamine concentration with carbon fiber electrode. Anal Chem 57:1518-1522.

Anderson CW, Cushman MR (1981) A simple and rapid method for making carbon fiber microelectrodes. J Neurosci Methods 4:435-436.

Armstrong-James M, Fox K, Millar J (1980a) A method for etching the tips of carbon fibre microelectrodes. J Neurosci Methods 2:431-432.

Armstrong-James M, Millar J (1979) Carbon fibre microelectrodes. J Neurosci Methods 1:279-287.

Armstrong-James M, Millar J, Kruk ZL (1980b) Quantification of noradrenaline iontophoresis. Nature 288:181-183.

Buda M, Gonon F, Cespuglio R, Jouvet M, Pujol JF (1981) In vivo electrochemical detection of catechols in several dopaminergic brain regions of anaesthetized rats. Eur J Pharmacol 73:61-68.

Budai D (1994) A computer-controlled system for post-stimulus time histogram and wind-up studies. J Neurosci Methods 51:205-211.

Budai D (2000) Microiontophoresis. http://members.aol.com/kations/iontophoresis/index.html

Cespuglio R, Faradji H, Riou F, Buda M, Gonon F, Pujol JF, Jouvet M (1981) Differential pulse voltammetry in brain tissue. II. Detection of 5-hydroxyindoleacetic acid in the rat striatum. Brain Res 223:299-311.

Clarencon D, Lestaevel P, Laval JD, Multon E, Gourmelon P, Buguet A, Cespuglio R (1999) Voltammetric measurement of blood nitric oxide in irradiated rats. Int J Radiat Biol 75:201-208.

Curtis DR (1964) Microelectrophoresis. In WL Nastuk, ed., Physical Techniques in Biological Research. New York: Academic Press, 144-190.

Darbon P, Michel V, Math F, Giorgi H, Machizaud F (1998) Immunoelectrodes in protein detection: comparison between glassy carbon and a semimetallic Ni/P thin film as binding support. Biological applications. Anal Chem 70:5072-5078.

Daws LC, Toney GM, Gerhardt GA, Frazer A (1998) In vivo chronoamperometric measures of extracellular serotonin clearance in rat dorsal hippocampus: contribution of serotonin and norepinephrine transporters. J Pharmacol Exp Ther 286:967-976.

Fox K, Armstrong-James M, Millar J (1980) The electrical characteristics of carbon fibre microelectrodes. J Neurosci Methods 3:37-48.

Friedemann MN, Robinson SW, Gerhardt GA (1996) o-Phenylenediamine-modified carbon fiber electrodes for the detection of nitric oxide. Anal Chem 68:2621-2618.

Fu J, Lorden JF (1996) An easily constructed carbon fiber recording and microiontophoresis assembly. J Neurosci Methods 68:247-251.

Garguilo MG, Michael AC (1996) Amperometric microsensors for monitoring choline in the extracellular fluid of brain. J Neurosci Methods 70:73-82.

Godwin DW (1993) A tungsten-in-glass iontophoresis assembly for studying input-output relationships in central neurons. J Neurosci Methods 49:211-223.

Gonon F, Buda M, Cespuglio R, Jouvet M, Pujol JF (1981) Voltammetry in the striatum of chronic freely moving rats: detection of catechols and ascorbic acid. Brain Res 223:69-80.

Haidarliu S, Shulz D, Ahissar E (1995) A multi-electrode array for combined microiontophoresis and multiple single-unit recordings. J Neurosci Methods 56:125-131.

Hellier M, Boers P, Lambert GA (1990) Fabrication of a metal-cored multibarrelled microiontophoresis assembly. J Neurosci Methods 32:55-61.

Hicks TP (1984) The history and development of microiontophoresis in experimental neurobiology. Prog Neurobiol 22:185-240.

Kasser RJ, Cheney PD (1983) Double-barreled electrode for simultaneous iontophoresis and single unit recording during movement in awake monkeys. J Neurosci Methods 7:235-242.

Kawagoe JL, Niehaus DE, Wightman RM (1991a) Enzyme-modified organic conducting salt microelectrode. Anal Chem 63:2961-2965.

Kawagoe KT, Jankowski JA, Wightman RM (1991b) Etched carbon-fiber electrodes as amperometric detectors of catecholamine secretion from isolated biological cells. Anal Chem 63:1589-1594.

Krnjevic K (1971) Microiontophoresis. In M Fried, ed., Methods of Neurochemistry. M. Dekker, New York.

Kruk ZL, Armstrong-James M, Millar J (1980) Measurement of the concentration of 5-hydroxytryptamine ejected during iontophoresis using multibarrel carbon fibre microelectrodes. Life Sci 27:2093-2098.

Kulagina NV, Shankar L, Michael AC (1999) Monitoring glutamate and ascorbate in the extracellular space of brain tissue with electrochemical microsensors. Anal Chem 71:5093-5100.

Kuras A, Gutmaniene N (1995) Preparation of carbon-fibre microelectrode for extracellular recording of synaptic potentials. J Neurosci Methods 62:207-212.

Kuras A, Gutmaniene N (2000) Technique for producing a carbon-fibre microelectrode with the fine recording tip. J Neurosci Methods 96:143-146.

Li BM, Mei ZT, Kubota K (1990) Multibarreled glass-coated tungsten microelectrode for both neuronal activity recording and iontophoresis in monkeys. Neurosci Res 8:214-219.

Malinski T, Taha Z (1992) Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. Nature 358:676-678.

Mermet C, Gonon FG, Stjarne L (1990) On-line electrochemical monitoring of the local noradrenaline release evoked by electrical stimulation of the sympathetic nerves in isolated rat tail artery. Acta Physiol Scand 140:323-329.

Millar J (1992) Extracellular single and multiple unit recording with microelectrode. In JA Stamford, ed., Monitoring neuronal activity: a practical approach. IRL Press, Oxford 1-27.

Millar J, Armstrong-James M, Kruk ZL (1981) Polarographic assay of iontophoretically applied dopamine and low-noise unit recording using a multibarrel carbon fibre microelectrode. Brain Res 205:419-424.

Millar J, O'Connor JJ, Trout SJ, Kruk ZL (1992) Continuous scan cyclic voltammetry (CSCV): a new high-speed electrochemical method for monitoring neuronal dopamine release. J Neurosci Methods 43:109-118.

Millar J, Williams GV (1988) Ultra-low noise silver-plated carbon fibre microelectrodes. J Neurosci Methods 25:59-62.

Navera EN, Sode K, Tamiya E, Karube I (1991) Development of acetylcholine sensor using carbon fiber (amperometric determination). Biosens Bioelectron 6:675-680.

Netchiporouk LI, Shram NF, Jaffrezic-Renault N, Martelet C, Cespuglio R (1996) In vivo brain glucose measurements: differential normal pulse voltammetry with enzyme-modified carbon fiber microelectrodes. Anal Chem 68:4358-4364.

Park JK, Tran PH, Chao JK, Ghodadra R, Rangarajan R, Thakor NV (1998) In vivo nitric oxide sensor using non-conducting polymer-modified carbon fiber. Biosens Bioelectron 13:1187-1195.

Ponchon JL, Cespuglio R, Gonon F, Jouvet M, Pujol JF (1979) Normal pulse polarography with carbon fiber electrodes for in vitro and in vivo determination of catecholamines. Anal Chem 51:1483-1486.

Rivot JP, Cespuglio R, Puig S, Jouvet M, Besson JM (1995) In vivo electrochemical monitoring of serotonin in spinal dorsal horn with Nafion-coated multi-carbon fiber electrodes. J Neurochem 65:1257-1263.

Rivot JP, Noret E, Ory-Lavollee L, Besson JM (1987) In vivo electrochemical detection of 5-hydroxyindoles in the dorsal horn of the spinal cord: the contribution of uric acid to the voltammograms. Brain Res 419:201-207.

Shigemitsu T, Nagata T, Matsumoto G, Tsukahara S (1980) Electrical properties of the carbon fibre electrode and its application. Med Biol Eng Comput 18:359-362.

Shigenaga T, Kato M, Taguchi K (1997) In vivo voltammetric studies of the effects of intrathecal morphine on noxious heat stimuli-induced serotonin release in the nucleus raphe magnus of anesthetized rats. Neurosci Res 29:257-262.

Shram NF, Netchiporouk LI, Martelet C, Jaffrezic-Renault N, Bonnet C, Cespuglio R (1998) In vivo voltammetric detection of rat brain lactate with carbon fiber microelectrodes coated with lactate oxidase. Anal Chem 70:2618-2622.

Shram NF, Netchiporouk LI, Martelet C, Jaffrezic-Renault N, Cespuglio R (1997) Brain glucose: voltammetric determination in normal and hyperglycaemic rats using a glucose microsensor. Neuroreport 8:1109-1112.

Stamford JA, Kruk ZL, Millar J (1984) A double-cycle high-speed voltammetric technique allowing direct measurement of irreversibly oxidised species: characterisation and application to the temporal measurement of ascorbate in the rat central nervous system. J Neurosci Methods 10:107-118.

Starrenburg AJ, Burger GC (1982) Carbon fiber as an electrode material. IEEE Trans Biomed Eng 29:352-355.

Suaud-Chagny MF, Cespuglio R, Rivot JP, Buda M, Gonon F (1993) High sensitivity measurement of brain catechols and indoles in vivo using electrochemically treated carbon-fiber electrodes. J Neurosci Methods 48:241-250.

Tamiya E, Karube I (1992) Ultramicrobiosensors for monitoring of neurotransmitters. Ann N Y Acad Sci 672:272-277.

Williams JEG, J. M, Kruk ZL (1992a) A comparison of cut and spark-etched electrodes for fast cyclic voltammetry. Br J Pharmacol 107.

Williams JEG, J. M, Kruk ZL (1992b) Preparation of spark-etched nodes for fast cyclic voltammetry. Br J Pharmacol 107.

Yao SJ, Xu W, Wolfson SK (1995) A micro carbon electrode for nitric oxide monitoring. Asaio J 41:M404-409.

Yavich L (1998) A new technique for measuring the temporal characteristics of the carbon fibre microelectrodes in in vivo voltammetry at millisecond time intervals. J Neurosci Methods 84:29-32.

Volume 45(1-4):75-77, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

SHORT COMMUNICATION

A color in situ hybridization method with improved sensitivity for the detection of low-abundance mRNAs

Beatrix Kovacs, Karoly Gulya*

Department of Zoology and Cell Biology, University of Szeged, Szeged, Hungary

ABSTRACT We have modified a nonradioactive (color) in situ hybridization method for the detection of mRNA populations, transcribed from the calmodulin (CaM) I gene, that exist in low abundances in tissues such as the white matter of the rat spinal cord. Our results indicate that increasing the pH of the hybridization solution from neutral to slightly alkaline (pH 8.0-8.5) drastically improves the detectable signal intensity of the digoxigenin-labeled CaM I gene-specific riboprobe while providing high spatial resolution. This method could be useful for the detection of other mRNA populations present in cells in low concentrations or in tissues where probe penetration might be impaired (*e.g.* a high lipid content).

Acta Biol Szeged 45(1-4):75-77 (2001)

KEY WORDS

alkaline pH
calmodulin
digoxigenin
gene expression
in situ hybridization
mRNA
spinal cord
rat
white matter

Calmodulin (CaM), an intracellular calcium receptor protein, is present in all eukaryotic cells and is involved in the regulation of numerous calcium-mediated cellular processes (Clapham 1995). The protein is encoded by multiple CaM genes (CaM I, II and III) that produce at least 7 transcripts. However, in consequence of the highly conserved sequences of the coding regions of these genes, their protein products are identical. The nervous tissue displays a widespread and differential distribution of the CaM mRNA populations in the brain under both normal (Ni et al. 1992; Solà et al. 1996; Palfi et al. 1999) and experimental conditions (Solà et al. 1997; Palfi and Gulya 1999; Vizi et al. 2000; Palfi et al. 2001). The CaM mRNA populations not only show region- and neuron type-specific distributions in the adult rat brain, but are differentially targeted to intracellular compartments of the neurons (Palfi et al. 1999). While CaM can be detected in glial cells, white matter areas of the brain and the spinal cord display little or no CaM mRNA expression. This could be due to the relatively low CaM mRNA concentrations in glial cells residing in these areas. Basyuk et al. (2000) reported an improved sensitivity when alkaline fixation preceded the nonradioactive in situ hybridization. In our present study, we investigated the possibility of improving the sensitivity of our color in situ hybridization method by using a modified alkaline hybridization solution to detect the CaM I mRNA populations present in low abundances in the white matter of the spinal cord.

Accepted May 29, 2001
*Corresponding author. E-mail: gulyak@bio.u-szeged.hu

Materials and Methods

The experimental procedures were carried out in strict compliance with the European Communities Council Directive (86/609/EEC), and followed the Hungarian legislation requirements (XXVIII/1998 and 243/1998) regarding the care and use of laboratory animals. Adult (200-220 g) male Sprague-Dawley rats were maintained under standard housing conditions. The animals were killed by decapitation, and their lumbar spinal cords were quickly removed, embedded in Cryomatrix embedding medium (Shandon Scientific Ltd., Pittsburgh, PA, USA) and frozen immediately at –70°C. Serial coronal cryostat sections (15 µm) were cut onto 3-aminopropyltriethoxy silane-coated glass slides, air-dried and stored at –70°C until further processing.

For the preparation of digoxigenin- (DIG)-labeled cRNA probes for in situ hybridization, the genomic sequence of the 3'-nonhomolog region of CaM I mRNA (Nojima and Sokabe 1987; Nojima 1989) was amplified by polymerase chain reactions (PCRs); sequence alignment was completed with the software BLASTN version 2.0.6 (Zhang and Madden 1997). PCRs were performed by employing EcoR I and BamH I restriction enzyme cleavage site-extended primers. The primer sequences complementary to rat genomic CaM I DNA were as follows: 5'-AGACCTACTTTCAACTACT, corresponding to the 30-48 bp sequence, and 5'-TGTAAAACTCATGTAGGGG, corresponding to the 237-255 bp sequence of exon 6 (Nojima and Sokabe 1987). Standard PCRs were run for 35 cycles (Palfi et al. 1998), and the resulting PCR product was cloned into a pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA) and sequenced (AB 373 DNA Sequencer, PE Applied Biosystems, Foster City, CA, USA) to confirm its identity. In vitro RNA syntheses

from the purified and linearized vector were carried out to prepare antisense and sense CaM I cRNA probes. The probes were synthesized with a DIG RNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany). The complementary probe sequence was 225 bp long.

Coronal cryostat sections from the lumbar spinal cord of the adult rat were fixed for 5 min in 2x SSC containing 4% formaldehyde, washed twice in 2x SSC for 1 min, and then rinsed in 0.1 M triethanolamine containing 0.25% acetic anhydride for 5 min at room temperature (RT). The sections were washed in 2x SSC for 5 min, dehydrated, air-dried and hybridized in 50 µl hybridization solution (50% formamide, 4x SSPE, 1x Denhardt's reagent, 10% dextran sulfate, 100 mM DTT, 0.1% SDS, 100 µg/ml salmon sperm DNA, 100 µg/ml yeast tRNA; the pH of the solution was adjusted to 8.5) containing 200 fmol/ml DIG-labeled probe. Hybridization was performed under parafilm coverslips in a humidified chamber at 55°C for 24 h. The sections were rinsed in 2x SSC at RT and 55°C for 5 and 10 min, respectively, then treated with RNase A (16 µg/ml) at 37°C for 30 min. The sections were washed in 2x SSC/50% formamide at 55°C for 2 x 10 min, and in 2x SSC at 55°C and RT for 10 min and 5 min, respectively. After posthybridization, the sections were washed in buffer B1 (100 mM Tris-HCl pH 7.5 and 150 mM NaCl) for 5 min, blocked in 5% heat-inactivated sheep serum in B1 for 2 h and incubated in sheep anti-DIG-alkaline phosphatase conjugate (Boehringer Mannheim; 1:1,000 dilution) in 5% sheep serum in B1 at 4°C for 24 h. Sections were washed in B1 for 3 x 5 min, and then in buffer B2 (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) for 10 min, and were developed in B2 containing 340 µg/ml nitro blue tetrazolium (NBT) and 180 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 24 h under darkroom conditions. The color reaction was stopped by rinsing the sections in a mixture of 10 mM Tris-HCl pH 8.0 and 1 mM EDTA for 5 min in RT, and the sections were then dehydrated and covered with Entellan (Merck, Darmstadt, Germany).

Results and Discussion

The conventional color in situ hybridization technique did not detect any cellular elements expressing CaM I mRNAs in the white matter area of the spinal cord (Fig. 1A). When our sensitive color in situ hybridization technique employing DIG-labeled riboprobe in a hybridization solution adjusted to slightly alkaline pH was used, widespread distribution of

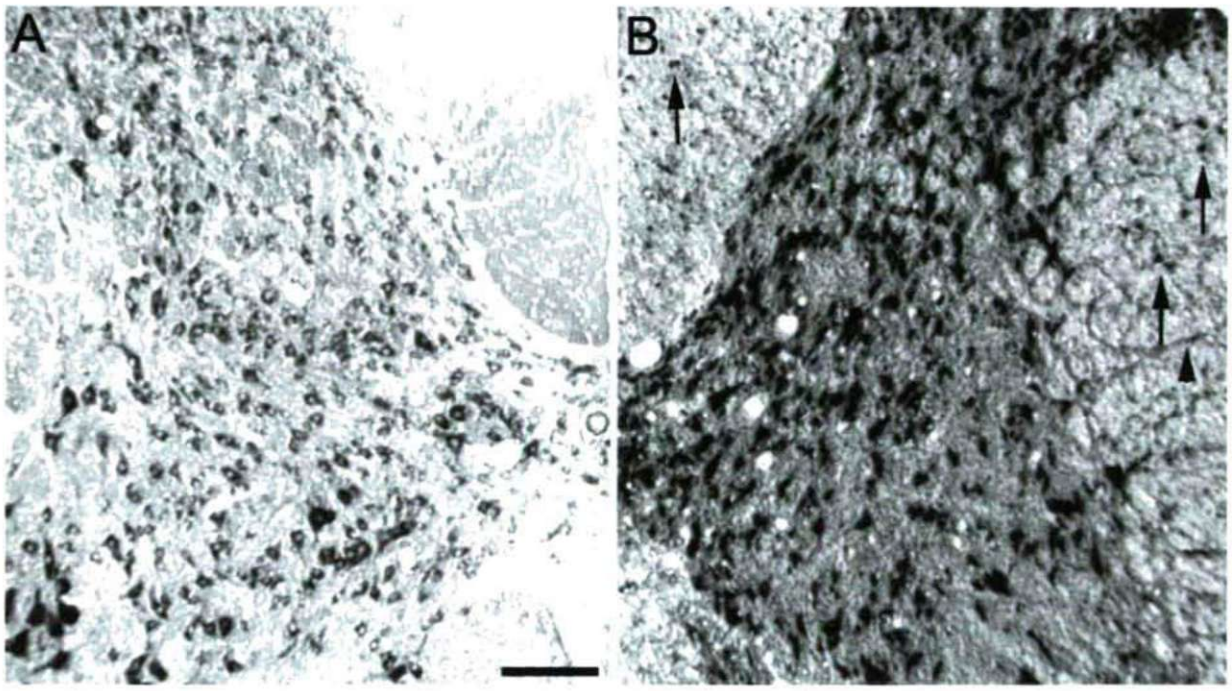


Figure 1. Nonradioactive in situ hybridization histochemistry of DIG-labeled CaM I-specific riboprobes in the rat lumbar spinal cord. **A.** Hybridization at neutral pH. CaM I mRNA is abundant only in the cells residing in the gray matter of the spinal cord, but completely absent in the white matter area. **B.** Hybridization at alkaline pH. Apart from the heavy labeling seen in the gray matter area, CaM I mRNA is widely expressed in small-to-medium-sized cells in the white matter area. Small cell somata (arrows) and sometimes a few processes (arrowhead) running radially to distances of 50-100 µm could be seen. Note that the neuropil of the gray matter is abundant in CaM I mRNA. Scale bar: 200 µm.

CaM I mRNA-expressing cells could be seen throughout the white matter area of the spinal cord (Fig. 1B). Many small and medium-sized cell bodies, probably glial cells, were heavily labeled in the dorsal, lateral and ventral funiculi of the white matter. A few of the labeled cells with fine processes could also be seen. The surrounding neuropil was also considerably labeled, especially in the dorsal column. DIG-labeled sense CaM I cRNA merely resulted in a level of labeling comparable to the background level (not shown).

The radioactive in situ hybridization technique, especially when combined with emulsion autoradiography (Palfi et al. 1999, 2000), provides a reasonably good spatial resolution, and is suitable, albeit cumbersome, method for the quantitative assessment of mRNA abundances. DIG-labeled riboprobes, however, are excellent choices for in situ hybridization histochemistries where high spatial resolution is needed, but where there is no option for quantitative measurements. Neither method provides an easy means of detecting mRNA populations present in low concentrations. To circumvent this problem, Basyuk et al. (2000) introduced alkaline fixation to in situ hybridization techniques in order to improve the sensitivity. Their studies indicated that alkaline formaldehyde dramatically increased (5- to 6-fold) the in situ hybridization signal with RNA probes. The signal increase was observed for the detection of both low and high-abundance messages. We adapted this trick to our color in situ hybridization protocols, and by using DIG-labeled riboprobes were able to detect CaM I mRNA populations in cells where their presence had previously escaped detection. Since alkaline fixation does not improve the retention of mRNA during in situ hybridization (Basyuk et al. 2000), but rather increases the accessibility of the target for the DIG-labeled cRNA probe, hybridization at alkaline pH is crucial for the increased sensitivity.

In summary, the protocol described here is easy to carry out and reliable, while retaining the high spatial resolution characteristic of the conventional DIG-based in situ hybridization technique, and can be applied to any mRNA class present in low quantities in a tissue.

Acknowledgments

We thank Dr. Arpad Palfi for his comments and Mrs. Susan Ambrus for her excellent technical help. This research was supported by the National Scientific Research Fund, Hungary (T 034621) and the Ministry of Health, Hungary (57/2000).

References

Basyuk E, Bertrand E, Journot L (2000) Alkaline fixation drastically improves the signal of in situ hybridization. Nucleic Acids Res 28:e46.

Clapham DE (1995) Calcium signaling. Cell 80:259-268.

Ni B, Rush S Gurd JW, Brown IR (1992) Molecular cloning of calmodulin mRNA species which are preferentially expressed in neurons in the rat brain. Mol Brain Res 13:7-17.

Nojima H (1989) Structural organization of multiple rat calmodulin genes. J Mol Biol 208:269-282.

Nojima H, Sokabe H (1987) Structure of a gene for rat calmodulin. J Mol Biol 193:439-445.

Palfi A, Gulya K (1999) Water deprivation upregulates the three calmodulin genes in exclusively the supraoptic nucleus of the rat brain. Mol Brain Res 74:111-116.

Palfi A, Hatvani L, Gulya K (1998) A new quantitative film autoradiographic method of quantifying mRNA transcripts for in situ hybridization. J Histochem Cytochem 46:1141-1149.

Palfi A, Vizi S, Gulya K (1999) Differential distribution and intracellular targeting of mRNAs corresponding to the three calmodulin genes in rat brain: a quantitative in situ hybridization study. J Histochem Cytochem 47:583-600.

Palfi A, Tarcsa M, Varszegi S, Gulya K (2000) Calmodulin gene expression in an immortalized striatal GABAergic cell line. Acta Biol Hung 51:65-71.

Palfi A, Simonka JA, Pataricza M, Tekulics P, Lepran I, Papp G, Gulya K (2001) Postischemic calmodulin gene expression in the rat hippocampus. Life Sci 68:2373-2381.

Solà C, Tusell JM, Serratosa J (1996) Comparative study of expression of calmodulin messenger RNAs in the mouse brain. Neurosci 75:245-256.

Solà C, Tusell JM, Serratosa J (1997) Calmodulin is expressed by reactive microglia in the hippocampus of kainic acid-treated mice. Neurosci 81:699-705.

Vizi S, Gulya K (2000) Multiple calmodulin genes exhibit systematically differential responses to chronic ethanol treatment and withdrawal in several regions of the rat brain. Mol Brain Res 83:63-71.

Zhang J, Madden TL (1997) PowerBLAST: new network BLAST application for interactive or automated sequence analysis and annotation. Genome Res 7:649–656.

Volume 45(1-4):79-82, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

SHORT COMMUNICATION

Skeleton of a dwarf from excavations

Gyula Farkas[1]*, Endre Nagy[2], Ferenc Kósa[3]

[1]Department of Anthropology, University of Szeged, [2]Department of Radiology, University Hospital, Szeged, [3]Department of Forensic Medicine, University of Szeged, Szeged, Hungary

ABSTRACT The authors describe the skeleton remains of a dwarf with achondroplasia dating from the 13th century. The finding is from a Hungarian site and can be found in the historical anthropological collection at the Department of Anthropology of the University of Szeged.

Acta Biol Szeged 45(1-4):79-82 (2001)

KEY WORDS
skeleton of a dwarf
findings from excavations
Hungarian site

Skeletons of dwarf individuals can rarely be found among skeletons dating from various archeological times. In 1964 there was a well-preserved dwarf skeleton finding from the 15th century found in Vajdaság (near Subotica, Yugoslavia) at Ludos-Csurgó site, which showed the symptoms of chondrodystrophia hyperplastica (Farkas and Lengyel, 1974).

There is another dwarf skeleton among the 16,000-piece collection at the Department of Anthropology of the University of Szeged, which will be discussed in this article. Unfortunately, this is an incomplete skeletal remain. However, as such skeletons are a curiosity in populations dating back several hundred years, this case may also be of interest from a medical historical point of view.

Materials and Methods

The skeletal remain was found east of the River Tisza (Eastern Hungary), at the Hortobágy-Máta-Szeghalomhatár site, on July 22, 1985, by archeologists Ibolya Nepper and György Módy, in a churchyard dated to the 13th century. It is a sporadic finding without a grave-number. It bears the record number 16,143 in the above-mentioned collection. The uncovered pieces are the following: a calvaria, left humerus, two femurs, left tibia, left ulna, right scapula, right clavicle, right hip and two ribs (Fig. 1).

Radiographs were taken of the five, more or less well preserved tubular bones (femur, tibia, humerus, ulna, clavicula). We used AGFA ORTHO CP-G PLUS 24x30 cm film, the intensifying screen pair was green-light ORTHO MEDIUM CURIX screens. The pictures were taken at 40 kV, 75 mA, 0.175 s exposure time.

Computer tomography (CT) was carried out with a Siemens Somatom 4 Plus machine, using spiral data collection. The parameters were the following: 120 kV, 170 mA, 1 mm thick laminas, 1 pitch. The reconstructions were made using Maximum Intensity Projection (MIP), Volume Rendering Technique (VRT) and Surface Shaded Display (SSD).

Results

As usually the case is with anthropological remains, first the age at death and sex of the skeleton was defined. The cranial sutures both on the endo- and ectocranial surfaces are completely open. The distal epiphyses on the two femurs and the caput humeri are not yet fused with the diaphysis. The two caput femoris and the great and small trochanters are not yet completely ossified, the surface of the crista iliaca is rough, not ossified. According to these attributes, the age at death was approximately 18-20 years.

The glabella is smooth (grade 0), the margo supraorbitalis is thin, the processus mastoideus moderately developed (grade 3), the tubera frontalia and parietalia are marked, the shape of the skull is rectangular. According to these characteristics, the individual should be identified as female.

There is sutural bone in the sutura lamboidea. The tuberositas deltoidea on the humerus is especially marked. The two collum femoris are short, the diaphysis of the right femur is thicker than that of the left, the two fovea capitis are deep, the fossa acetabuli shallow.

There are few data available for the metric characterisation of the finding. The maximal width of the skull is 165 mm, the fronto-temporal width is 108 mm. In the norma verticalis, the shape of the neurocranium is spheroidal. The length of both femurs is 210 mm (without the distal epiphysis), the maximal length of the left tibia is 196 mm, the transversal diameter of the caput femoris is 43 mm, the left is 41 mm, the circumference of the diaphysis of the right femur is 86 mm, the left is 79 mm.

Based on the available data we tried to assess the height of the individual. Because of the exceptionally short long

Accepted May 4, 2001
*Corresponding author. E-mail: farlgy@bio.u-szeged.hu

bones of the skeleton, the most widely used Bach-Breitinger and Trotter-Gleser methods for estimating height in adult skeletons could not be used. Moreover, the distal epiphyses of the long bones were also missing. Thus, we used Pearson's method (Martin and Saller 1957), which offers eight ways for assessment of height. From these only three can be used in case of a female skeleton. The results are the following: 1) height = 69.154 + 1.126 x (length of femur + length of tibia) = 114.9 cm; 2) height = 72.844 +1.945 x length of femur = 113.7 cm; 3) height = 69.561 + 1.117 x length of femur + 1.125 x length of tibia = 115.1 cm. As the distal epiphyses of the femurs were missing, we had to assume that the individual was taller than our results (114-115 cm) show. If we suppose that the missing condyle was 2 cm long, the maximal height of the individual must have been around 117-118 cm.

We also carried out radiological tests on the finding. The slightly incomplete os frontale, both ossa parietale and also a small part of the os occipitale were pieced together in the right position, and axial, anterioposterior and lateral X-rays were taken, as well as of the piece of calvaria, including the incomplete left os temporale. Later on the bones were also subjected to CT (Fig. 2). The structure of tubular bones can be perfectly assessed on traditional radiographs. Bones are wide but short. The V-shaped proximal epiphyseal plate of the stubby tibia is very characteristic of achondroplasia. (Fig. 3). Judging from the occasionally present epiphyseal plate zones and the well-developed points of muscle adhesion makes it likely that the skeleton belonged to a young adult.

Summing up the results from the traditional radiographic pictures and the different sort of reconstructions by spiral CT carried out on the skull fragments, we can say that for the examination of the bone structure the traditional X-ray resulted a picture of much better quality. Whereas the the 3D CT reconstruction, using the postprocessing functions of digital imagery, made it possible to study the whole of the cranial bones from different angles. In theory, it is possible to reconstruct the whole skull with the help of a special software.

As opposed to the usual descriptive anatomical and anthropometrical examinations, the instrumental analysis of the

Figure 1. The skeletal remain found at the Hortobágy-Máta-Szeghalomhatár findspot.

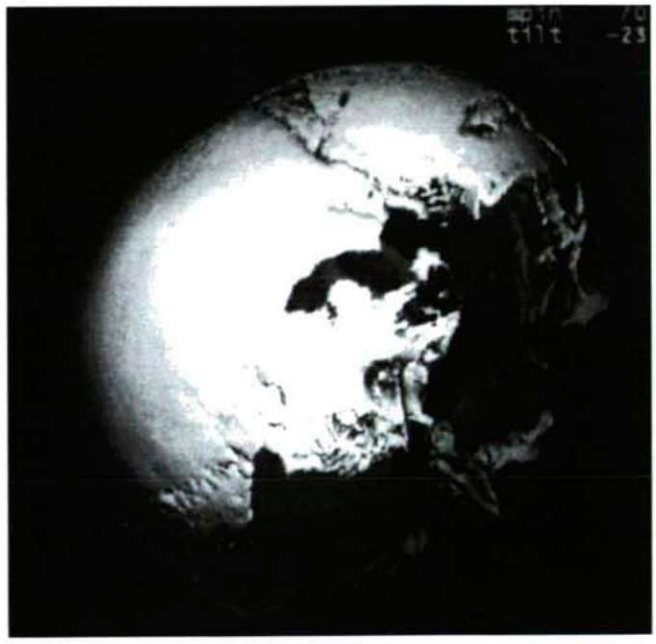


Figure 2. CT scan of the skull.

Figure 3. See Results for details.

fine structure of bones help to learn about the pathological deformations in anthropological finds. They also present a new opportunity for research in case of developmental anomalies influencing longitudinal growth. That is why we thought it was worthwhile to carry out these modern radiological tests on these skeletal finds.

Discussion

In case of sporadic finds the taxonomic examinations and the appraisal of the results may raise a number of problems, especially when the height and age at death of an individual of abnormal growth (dwarf) must be assessed. Determining height based on the length of the limb bones in an individual suffering from bone formation disorder is especially difficult, as the index numbers and regressive diagrams were determined based on bone size and height in normal populations. The usual formulas can only result in a rough estimate in height when examining a dwarf suffering from chondrodystrophy. Unfortunately, there is no index number available for pathologists and paleopathologist to use when assessing the size of limb bones which could be used in such cases instead of the current practice of rough estimation.

Figure 4. See Results for details.

Most certainly, there are other methods for the assessment of height and age at death than the position of epiphysial lines in limb bones, their distance from the marrow cavity, the structure of cancellous tissue in the epiphysis, the level of ossification in sutures, etc (Nemeskéri et al. 1960).

The modern X-ray diagnostic methods can also help historical anthropological research to construct the missing pieces of a fragment skull with the help of a software. A fragment skull of unknown origin could be compared to a painting or photograph of a known person for the purposes of identification. Imaging radiological methods and identification techniques using superimposition open up new territories both in forensic and historical anthropology (Iscan and Schaaffhausen 1875, 1883; His 1895a, b; Grüner 1901; Stadtmüller 1948; Grüner and Reinhard 1959; Fikentscher 1978; Hunger and Leopold 1978; Welcker 1883; Helmer 1984).

Archeological and historical anthropological studies have shown that skeletons of dwarfs with dysplasia can be found in most ancient societies and cultures (Egyptian, Precolumbian, Greek, and other European) (Seligmann 1912; Keith 1913; Priesel 1920; Bleyer 1940; Brothwell 1967; Brothwell and Sandison 1967; Fakas and Lengyel 1974; Hoffmann 1976; Orthner and Putschar 1985). The skeleton finds do not show any signs of violence indicating that ancient and medieval societies treated these "handicapped" people with sympathy, they had a place in society. This assumption is also supported by another Hungarian find. An adult skeleton dated to the 11-16th century was uncovered in a churchyard in Bátmonostor which had the femur and tibia united at right angles as a result of an infectious disease (Fig. 4). The individual would have been unable to walk without an aid (a wooden "artificial leg" which could be attached to his own), still, society did not expel him.

We also agree that these results are correct as far as cultural history is concerned, which is also supported by the results of paleopathological examinations, namely that in these cases the cause of death was most probably natural.

References

Bleyer A (1940) The Antiquity of Achondroplasia. Annls Med Hist 2:306-307.

Brothwell DR (1967) Major Congenital Anomalies of the Skeleton: Evidence from Earlier Populations. In Brothwell DR, Sandison AT, eds., Diseases in Antiquity: A Survey of the Diseases, Injuries and Surgery of Early Populations. Springfield, IL, 423-446.

Brothwell DR, Sandison AT, eds., (1967) Diseases in Antiquity: A Survey

of the Diseases, Injuries and Surgery of Early Populations. Springfield, IL.

Farkas Gy, Lengyel I (1974) Skeleton of a medieval dwarf from Ludos-Csurgó (Yugoslavia). Móra Ferenc Múzeum Évkönyve, Szeged, 1971/2:209-212.

Fikentscher H (1978) Der heutige Stand der Forschung über Friedrich Schillers sterbliche Reste. Personal communication.

Grüner O (1961) Bemerkungen zur photographischen Identifizierung menschlicher Schädel. Beitr Gerichtl Med 21:149-155.

Grüner O, Reinhard R (1959) Ein photographisches Verfahren zur Schädelidentifizierung. Dtsch Z Gerichtl Med 48:247-256.

Helmer R (1984) Schädelidentifizierung durch elektronische Bildmischung. Zugleich ein Beitrag zur Konstitutionsbiometrie und Dickenmessung der Gesichtsweichteile, Kriminalistik Verlag, Heidelberg.

Helmer R, Grüner O (1977) Vereinfachte Schädelidentifizierung nach dem Superprojektionsverfahren mit Hilfe einer Video-Anlage. Z Rechtsmedizin 80:183-187.

His W (1895a) Johann Sebastian Bach. Forschungen über dessen Grabstätte, Gebeine und Antlitz. Bericht an den Rath der Stadt Leipzig. FCW Vogel, Leipzig.

His W (1895b) Anatomische Forschungen über Johann Sebastian Bach's Gebeine und Antlitz nebst Bemerkungen über dessen Bilder. Abh Math-Physical KI Kgl Sachs Ges Wiss.

Hoffmann JM (1976) An Achondroplastic Dwarf from the Augustine Site (CA-Sac-127). Contributions of the University of California Archaeological Research Facility 30:65-119.

Hunger H, Leopold D (1978) Personenerkennung durch Superprojektion. In Hunger H, Leopold D, eds., Identifikation. Springer Verlag, Berlin, 263-286.

Iscan MY, Helmer RP, eds., (1993) Forensic Analysis of the Skull. Craniofacial Analysis, Reconstruction and Identification. Wiley, New York.

Keith A (1923) Abnormal Crania - Achondroplastic and Acrocephalic. J Anat Phys 47:189-206.

Martin R, Saller K (1957) Lehrbuch der Anthropologie. Bd 1. Fischer G Verlag, Stuttgart.

Nemeskéri J, Harsányi L, Acsádi Gy (1960) Methoden zur Diagnose des Lebensalters von Skelettfunden. Anthrop Anz 24:70-95.

Ortner DJ, Putschar WGJ (1985) Identification of Pathological Conditions in Human Skeletal Remains, Smithsonian Inst Press, Washington.

Priesel A (1920) Ein Beitrag zur Kenntnis des Hypophysären Zwergwuchses. Beitr Path Anat Allg Path 67:220-274.

Schaaffhausen H (1875) Über die Totenmaske Shakespeares. Jahrb Dtsch Shakespeare Ges 10:26-49.

Schaaffhausen H (1883) Der Schädel Raphaels. Zur 400jährigen Geburtstagsfeier Raphael Santi's. Bonn, Max Cohen and Son.

Seligmann CG (1912) A Cretinous Skull of the Eigteenth Dynasty. Man 12:17-18.

Stadtmüller F (1948) Über Schädelidentifikation, insbesondere die zeichnerische Zugehörigkeitsprüfung. Grenzgeb med 1:63-68.

Welcker H (1883) Schiller's Schädel und Todtenmaske. Nebst Mitteilungen über Schädel und Todtenmaske Kant's. Viehweg and Son, Braunschweig.

Volume 45(1-4):83-84, 2001
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS-

OBITUARY

Dr. Pál Lipták (1914-2000)

On July 6, 2000, Pál Lipták, professor emeritus, passed away in Budapest.

He was born in Békéscsaba on February 14, 1914. He attended secondary school in his hometown; between 1932-1937 he studied natural history and geography at Pázmány Péter University in Budapest, where he eventually took a teachers' degree in 1937. From 1934 on he was a member of Eötvös József Collegium. At the same university he obtained doctorate "summa cum laude" in the arts in 1938 with his dissertation entitled "The Geography of Békéscsaba." From September 1, 1938, he taught at the teacher training college at Miskolc. In 1939 he joined the army for a two-year compulsory military service, after which, in 1941, he got a teaching position at the public Teachers' College in Budapest. In 1943 he was elected teacher in "Fasori" Secondary School in Budapest.

In April 1944 he joined the army again, and in May he was taken captive by Soviet troops to return home in 1948 only. Afterwards he taught at a primary school, the successor of "Fasori" Secondary School. During the summer of 1949 he worked as a scientific offical, then as a research-fellow for the Anthropological Collection of the Museum of Natural History, where he continued the work interrupted by the war. In March, 1956, he became Candidate of Biological Sciences by defending his thesis "The Major Questions of Anthropology in the Territory between the Danube and the Tisza Rivers between the 7th and 13th Centuries AD."

On March 16, 1960, he was appointed head of the Department of Anthropology at József Attila University, Szeged. In January, 1969, he defended his doctoral thesis "The Anthropology of Hungarian Ethnogenesis" and became Doctor of Sciences. In July, 1969, he was appointed professor. He retired from the department in Szeged in the summer of 1980.

His research was focused on questions of historical anthropology, especially Hungarian ethnogenesis. His work was characterised by a well-founded specialization. In the greater part of his hundred scholarly publications he dealt with the analysis and synthesis of historical anthropological finds. His attention was focused on the periods of Hungarian migration, the Hungarian Conquest and the Arpads and Hungarian ethnogenesis in this context. Based on examinations on skeletons, he improved the method of anthropotaxonomical differential diagnosis for europids and mongolids. In 1983 he published his book entitled "Avars and Ancient Hungarians."

In a few monographies on local history he co-authored communications on the anthropology of the living Hungarian population.

He took short field-trips abroad and participated in congresses. He was a member of the Anthropological Theme Committee of the Hungarian Academy of Sciences (1958-1962), later on its Committee (1962-1985), and the editorial board of Antropológiai Közlemények (Communications in Anthropology) (1957-1992), and was the editor-in-chief of Acta Biologica Szegediensis (1975-1980).

As part of his academic work he wrote a textbook for university students entitled "Anthropology and the Evolution of Man," which was the first of its kind. Several of his students earned their doctoral degrees under his supervision. In 1989 he was awarded the Bartucz Lajos Commemorative Medal, and in February, 1994, the title of "professor emeritus" by József Attila University.

Pál Lipták's death means that we lost the last member of the excelling generation of Hungarian anthropologists (Mihály Malán, János Nemeskéri, Miklós Fehér) trained in the mid-1930s.

His work has left a lasting mark on the face of Hungarian anthropology.

From Pál Lipták's major communications

Anthropologische Beiträge zum Problem der Ethnogenesis der Altungarn. (1951) Acta Arch Hung 1:231-249.

L'analyse typologique de la population de Kérpuszta au Moyen Age. (1953) Acta Arch Hung 3:303-370.

Zur Frage der Anthropologischen Beziehungen zwischen dem Mitteler Donaubecken und Mittelasien (1955) Acta Orient Hung 5:271-312.

Data to the Bronze-Age Anthropology of the Territory between the Danube and Tisza Rivers (1957) Anthrop Közl 1:3-16.
Awaren udn Magyaren im Donau-Theiss Zwischenstromgebiet (1958) Acta Arch Hung 8:199-268.
The Avar period Mondoloids in Hungary. (1959) Acta Arch Hung 10:250-279.
Anthropology and Historical Anthropology (1959) Anthrop Közl 3:111-120.
Die Bedeutung der taxonomischen Fragen in der historishcen Anthropologie (1961) Acta F.R.N. Univ Comen 5:309-314.
Anthropology and the Evolution of Man. Textbook for University Students, 1962, Budapest, p. 228.
Homo Sapiens - species collectiva. (1962) Anthrop Közl 6:17-27.
On the taxonomic method in Palaeoanthropology /historical anthropology/ (1965) Acta Biol Szeged 11:169-183.
Über die Anthropologie der Bevölkerung des südlichen Teils der ungarischen Tiefebene in der Arpadenzeit (Gyula Farkas, co-auth.), 1968, Móra Ferenc Múz Évk 2:135-141.
Anthropology and the Evolution of Man. Textbook for University Students. (1969) Tankönyvkiadó, Budapest, p. 284.
The Paleoanthropology of Hungarian Ethnogenesis. Theses of Doctoral Dissertation (1970) Anthrop Közl 14:85-94.
Physical anthropological examination of a cemetery in Mokrin from the early Bronze Age. (Gyula Farkas, co-auth.), 1971, Diss et Monogr 11. Beograd 239-271.
A critical review of paleoanthropological studies of the Avars in Hungary. (1970) Acta Biol Szeged 16:117-127.
Origin and development of the Hungarian people on the basis of anthropological remains. (1975) Hung Pas 4:79-94.
Anthropologische Auswertung des brozezeitlichen Gräberfeld bei Tápé (Gyula Farkas, co-auth.). (1975) Fontes Arch Hung 229-267.
Avars und Ancient Hungarians. Akadémiai Kiadó, Budapest (1983) p. 208.

On His Life and Works

In Vol. 9., 10., 12.,13., 16., 17., 20., 22., 25., 27., of Anthrop Közl in the bibliography of Hungarian anthropology.
Szeged University Almanach. 1921-1970. Szeged. 1971, p. 190.
Anthrop Közl 28; 1984 3-6. Acta Biol Szeged. 35; 1989, pp. 3-7.
Farkas, Gyula L. - Dezső, Gyula: The History of Hungarian Anthropology from the beginning up today. Szeged, 1994, pp. 88-89.
Szeged University Almanach. 1921-1995. Szeged, 1996, p. 360.

Instructions to Authors

Submission of manuscripts

Submission of a manuscript to Acta Biologica Szegediensis automatically involves the assurance that it has not been published and will not be published elsewhere in the same form. Manuscripts should be written in English. Since poorly-written material will not be considered for publication, authors are encouraged to have their manuscripts corrected for language and usage by a trusted expert.

There are no explicit length limitations: a normal research article will occupy 4-6 printed pages; reviews might be considerably longer. Authors should submit three sets of the complete manuscript and illustrations, together with a computer disk containing an electronic version of their manuscript. The elctronic file is considered the final material. Both Macintosh and PC versions will be accepted. The disk should be labeled with the date, the first author's name, the file name of the manuscript and the software, disk format and hardware used. Acta Biologica Szegediensis will not return copies of submitted manuscripts and figures. Requests to return original figures will be honored as a courtesy, but cannot be guaranteed. If instructions are not followed, authors will be asked to retype their manuscripts.

Manuscript format

Only good-quality laser printouts will be accepted. All pages should be printed with full double spacing, 2.5 cm margins, and a nonjustified right margin. A standard 12 point typeface (e.g. Times, Helvetica or Courier) should be used throughout the manuscript, with symbol font for Greek letters. Boldface, italics or underlined text should not be used anywhere in the manuscript. Footnotes are not permitted. Each page should be numbered at the bottom as follows:

Page 1. Title page: Complete title, first name, middle initial, last name of each author; where the work was done (authors' initials in parentheses if necessary); mailing address, phone, fax, and e-mail of the corresponding author; a running title of no more than 48 characters and spaces.

Page 2. Abstract: no more than 200 words, followed by 4-6 key words.

Beginning on page 3: Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, Figure Legends, Tables. Each section should be begun on a new page.

The manufacturer's name and location should be given in parentheses for reagents and instruments. Sources for all antibodies and nucleotide sequences should be indicated. Customary abbreviations in common use need not be defined in the text (e.g. DNA or ATP). Other abbreviations should be defined the first time that they are used. Quantitative results must be presented as graphs or tables and supported by appropriate experimental design and statistical tests. Only SI units may be used. For studies that involve animals or human subjects, the institutional, national or international guidelines that were followed should be indicated.

References

Only work that has been published or is in the press may be referred to. Personal communications should be acknowledged in the text and accompanied by written permission. In the text, references should be cited by name and year, e.g. Bloom (1983) or (Schwarz-Sommer et al. 1990) or (Maxam and Gilbert 1977). In the References, references should be listed alphabetically by first authors (including all co-authors) and chronologically for a given author (beginning with the most recent date of publication). Where the same author has more than one publication in a year, lower case letters should be used (e.g. 1999a, 1999b, etc.). Periods should not be used after authors' initials or abbreviated journal titles (e.g. Acta Biologica Szegediensis should be cited as Acta Biol Szeged). Inclusive page numbers should be used. Examples:

Bloom FE (1983) The endorphins: a growing family of pharmacologically pertinent peptides. Annu Rev Pharmacol Toxicol 23:151-170.

Coons AH (1978) Fluorescent antibody methods. In Danielli JF, ed., General Cytochemical Methods. Academic Press, New York, 399-422.

Maxam AM, Gilbert WA (1977) A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560-564.

Monod J, Changeux J-P, Jacob F (1963) Allosteric proteins and cellular control systems. J Mol Biol 6:306-329.

Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H (1990) Genetic control of flower development by homeotic genes in Antirrhinum majus. Science 250:931-936.

Illustrations

Three complete sets, including a high-quality "original" for publication, must be submitted with the manuscript. The back of each figure or composite plate should be labeled in soft lead pencil, indicating the orientation, the figure number, and the first author's name. The back of the best set should be marked "use for reproduction" or "original". Authors are encouraged to submit digital images of photographs, line drawings or graphs for printing. Most major image editing and drawing/illustrator computer software files (both Macintosh and PC) in TIFF or EPS formats are acceptable. It is particularly important that adequate resolution (at least 300 dpi, preferably 600 dpi) is used in making the original image.

Figure legends

Figures should be numbered consecutively with Arabic numerals. Material in the text should not be duplicated and methods should not be described. The size of scale bars should be indicated when appropriate. The first figure in the text should be referred to as Fig. 1, and so on.

Tables

Tables should be numbered consecutively with Arabic numerals. A brief title should be included above the table. Each table should be printed double spaced, without vertical or horizontal lines, and on a separate sheet. Material in text should not be duplicated and methods should not be described. The first table in the text should be referred to as Table 1, and so on.